Functional antibodies targeting IsaA of *Staphylococcus aureus* augment host immune response and open new perspectives for antibacterial therapy

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ABSTRACT

Staphylococcus aureus is the most common cause of nosocomial infections. Multiple antibiotic resistance and severe clinical outcomes provide a strong rationale for development of immunoglobulin-based strategies. Traditionally, novel immunological approaches against bacterial pathogens involve antibodies directed against cell surface exposed virulence-associated epitopes or toxins. In this study, we generated a monoclonal antibody targeting the housekeeping protein IsaA, a suggested soluble lytic transglycosylase of S. aureus and tested its therapeutic efficacy in two experimental mouse infection models. A murine anti-IsaA antibody of IgG1 subclass (UK-66P) showed highest binding affinity in Biacore analysis. This antibody recognizes all S. aureus strains tested including hospital-acquired and community-acquired methicillin-resistant S. aureus strains. Therapeutic efficacy in vivo was analyzed in mice using a central-venous catheter-related infection model and a sepsis survival model. In both models anti-IsaA IgG1 conferred protection to staphylococcal infection. Ex vivo, UK-66P activates professional phagocytes and induces highly microbicidal reactive oxygen metabolites in a dose-dependent manner, resulting in bacterial killing. The study provides proof of concept that monoclonal IgG1 antibodies with high-affinity to the ubiquitous expressed single epitope IsaA are effective in the treatment of staphylococcal infection in different mouse models. Anti-IsaA antibodies might be a useful component in an antibody-based therapeutic for prophylaxis or adjunctive treatment of human cases of S. aureus infections.

Keywords: Staphylococcus aureus; antibodies, immunotherapy; phagocytosis

INTRODUCTION

Staphylococcus aureus is a nosocomial and community-acquired pathogen, which causes several diseases, ranging from minor skin infections to serious life-threatening wound infections, bacteraemia, endocarditis, pneumonia and toxic shock syndrome (25). The potential of S. aureus to develop multidrug resistance to traditional antibiotics has created renewed interest in using alternative treatment options such as antibody-based immunotherapy approaches (5, 19, 31, 34). The key factor for developing an anti-staphylococcal immunotherapy depends on the identification of those bacterial antigens expressed in vivo that provide protection by the immune system of a wide patient population during infection (9). Therefore, several studies have investigated the immune response to S. aureus to determine which bacterial antigens are associated with protective anti-staphylococcal antibodies (4, 7, 9, 24, 27, 39). However, the significance and specificity of the immune response in S. aureus infections proved difficult to be elucidated as a number of clinical trials have recently failed (34). Other immunotherapy approaches target typical virulence factors, which may play a central role in the pathogenesis of staphylococci (2, 3, 10, 12, 18, 20, 22, 27, 37, 43, 44). But, functional redundancy of adhesion proteins or the appearance of escape mutants may limit the efficacy of strict monovalent immunotherapeutic strategies. Some evidence suggests that also bacterial cell wall components with immunogenic properties can serve as potential candidates for immunotherapy development (16, 21).

One such protein involved in cell wall metabolism is the immunodominant staphylococcal antigen A (IsaA). IsaA is a highly immunogenic, non-covalently cell wall bound, lytic transglycosylase (24, 36, 38) which is co-regulated with a glycylglycine endopeptidase, LytM (8). Strains of S. aureus lacking IsaA expression are viable and the paralogue SceD, a second lytic transglycosylase, is able to

compensate the loss (38). All those pieces of evidence implicate a role of IsaA as complex regulated factor involved in cell wall growth and division. Hence, the IsaA antigen appears not to be a typical virulence factor but rather a standard cellular housekeeping protein.

The present study was conducted to further clarify the therapeutic potential of antibodies to S. aureus with a particular focus on IsaA as target. We recently developed an animal model of S. aureus catheter induced sepsis in immunocompetent mice that closely mimics the clinicopathological features of human disease (23). Applying this experimental system and a sepsis survival model in mice, the immunotherapeutic potential of a murine monoclonal antibody recognizing IsaA was investigated. Both infection models show that the passive anti-IsaA antibody application significantly reduces the bacterial burden in host tissues compared to untreated animals. In addition, anti-IsaA immunotherapy triggers highly microbicidal reactive oxygen metabolites by phagocytes and killing of S. aureus.

Overall, the data presented within the study prove that the staphylococcal immunodominant antigen IsaA is a promising candidate for antibody-based therapy in humans that could significantly improve the outcome of S. aureus infection.

MATERIALS AND METHODS

Monoclonal antibody production

Murine monoclonal antibodies were generated by standard protocol of Synaptic Systems (Goettingen) using ELISA and Western blot screening (see also www.sysy.com/mabservice.html). Briefly, three 8 to 10 weeks old female Balb/c mice were immunized over a period of 17 days with the purified recombinant IsaA (rIsaA) protein. Cells from knee lymph nodes were fused with the mouse myeloma cell line P3X63Ag.653 (ATCC CRL-1580). The hybridoma elected in this study was cloned two times by limiting dilution. The monoclonal antibody was determined to be of the IgG1 subclass. The IgG1 antibody solution was purified by Protein G Fast Flow affinity chromatography as described elsewhere (17). Purified antibody anti-IsaA IgG1 mAb (UK-66P) and murine isotype control antibody were further used.

Biosensor measurements

To determine the affinity of the monoclonal antibody UK-66P to IsaA the kinetics of binding of rIsaA to immobilized antibody was determined by means of label-free surface plasmon resonance using the BIACORE®2000 system (GE Healthcare Europe GmbH, Freiburg, Germany). Reversible immobilization of the antibody UK-66P was performed using an anti mouse Fc antibody covalently coupled in high density (18,700 resonance units RU) to a CM5 sensor surface according to manufacturer's instructions (Mouse Antibody Capture Kit, GE Healthcare). The average amount of captured antibody UK-66P onto the anti mouse Fc surface corresponds to about 640 RU. A blank anti-mouse Fc surface was used as control surface for monitoring unspecific binding and performing reference subtraction. Interaction analyses were performed using HBS-EP buffer (10 mM HEPES pH7.4,

150 mM NaCl, 3 mM EDTA, 0.005% Tween 20). Sensorgrams were recorded at a flow rate of 30 µl/min at 25°C. Association and dissociation times were set to 3 and 15 min, respectively. The anti-Fc capturing surfaces were regenerated after each cycle using short pulses of 10 mM glycine pH 1.7. Affinities (K_D) and rate constants for association (k_a) and for dissociation (k_d) were calculated using the BIAevaluation software 4.0.1 fitting the obtained sensorgrams to a 1:1 Langmuir binding model.

Bacteria

The wild-type *S. aureus* strain MA12 and its isogenic IsaA insertion mutant strain MA12 ΔisaA::Em^r have been continuously used. The IsaA mutant phenotype strain served as an internal control for UK-66P specificity within the ex vivo and in vivo experiments. The protein A mutant from wild-type strain Cowan I Δspa::Tc^r (DU 5889) was used to test cross-reactivity of UK-66P. The strains ANS46 (SCC mec III), BK2464 (SCCmec II), HDE288 (SCCmec IV), MU50 (vancomycin-resistant S. aureus (VRSA)), MW2 (CA-MRSA) and EMSRA-15 (epidemic MRSA) served as additional controls for binding experiments. The strain USA300 (CA-MRSA) was also used for survival analysis. Single colonies of the respective strain were used to inoculate a 25 ml 2xYT broth culture overnight at 37°C. The culture was washed in PBS and serial dilution was performed to obtain a concentration of 10^6 or 10^7 colony-forming units (cfu), respectively that was confirmed by quantitative culture analysis. The bacteria were suspended in 0.1 ml or 0.5 ml of physiologic NaCl solution for infection experiments (see below).

Indirect immunofluorescence assay

S. aureus MA12, its isogenic IsaA insertion mutant strain MA12 ΔisaA and Cowan I Δspa were grown in TSB to mid-log phase (OD_{600} 0.5), and 1 ml of the

culture was centrifuged for 5 min at 13,000 x g. The washed bacterial sediment was suspended in 1 ml PBS (10 mM sodium phosphate (pH 7.2), 0.15 M sodium chloride). An aliquot of the cell suspension (100 µl) was mixed with UK-66P in PBS and incubated at room temperature for 15 min. Then five µl of FITC-conjugated goat anti-mouse IgG (H+L chain) (Dianova, Hamburg, Germany) was added, and the mixture was incubated at room temperature for 30 min in a dark and moisture chamber. After the bacteria were washed three times with 200 µl of PBS, the cells were suspended in 100 µl of PBS and viewed with a ZEISS Axioplan epifluorescence microscope. The images were captured with the Low light camera INTAS MP Focus 5000. The fluorescence and the phase contrast images were processed in Adobe Photoshop CS2.

Quantitative determination of neutrophil activation and oxidative burst

For the quantitative determination of murine neutrophil oxidative burst, the commercially available flow cytometry based Phagoburst® test kit was used according to the manufacturer`s instruction (ORPEGEN Pharma, Heidelberg, Germany). The Phagoburst ® assay allows the determination of neutrophils which oxidize the fluorogenic substrate dihydrorhodamine (DHR) 123. For oxidative burst analysis, heparinised murine blood was drawn from mice by femoral vein puncture under general anaesthesia. Wild-type S. aureus strain MA12 and IsaA mutant strain MA12 ΔisaA were cultured in LB-Medium at 37°C and harvested in mid-logarithmic phase. Bacteria were washed twice with PBS and adjusted to $1x10^9$ cfu/ml. Blood cells were stimulated with 20 µl of bacteria. Before challenge of neutrophils, bacteria were opsonized with different dilutions of UK-66P antibody (0.3 mg/ml or 0.6 mg/ml, respectively) or isotype control antibody (IC; dose equivalent) for 20 min at room temperature. In a forward/side scatter dot plot, gate was set on granulocytes. The

mean fluorescence intensity (MFI) correlating with oxidation quantity per individual neutrophil (oxidative burst) and the percentage of neutrophils (recruitment) having produced reactive oxygen metabolites were analyzed. For that purpose, a negative control sample was used to set a marker (M1) for fluorescence-1 (FL1) so that less than 1% of the events were positive. In the study samples, the numbers of events above this marker position were counted. Cells were analysed with a FACSCalibur flow cytometer using CellQuestPro and WinMDI 2.9.

Neutrophil intracellular survival assay

Intracellular survival assays were performed as follows. Bacterial cultures were washed twice in PBS, adjusted to 5×10^{7} cfu and mixed with 100 µl whole mouse blood and then incubated at 37°C in a water shaker. Before co-incubation, bacteria were opsonized with UK-66P antibody (0.6 mg/ml) or isotype control antibody (IC, dose equivalent) for 20 min at room temperature. Gentamicin (final concentration 400 µg/ml) and lysostaphin (final concentration 100 µg/ml) were added after 45 min to kill extracellular bacteria. At 60 min, the content of samples were withdrawn, centrifuged to pellet the neutrophils, and washed to remove the antibiotic medium. Neutrophils were then lysed in 1% saponin, and cfu calculated by plating on TSB.

Catheter-related generalized infection in mice

The Ethics Committee of the Lower Franconia authorities endorsed all animal studies. Age, gender and weight matched NMRI mice (Charles River, Sulzfeld, Germany) were used in the experiment. Mice were intraperitoneally anaesthetised with xylazin (8 mg/kg body weight) and ketamine (100 mg/kg body weight) and a central venous catheter was surgically placed as already described (23). Twenty-four

hours after surgery the mice were inoculated via the catheter with 100 µl of a S. aureus suspension, containing $1x10^7$ cfu S. aureus bacteria. The bacterial suspension was allowed to dwell within the catheter lumen for 15 min. The content of the catheter was then flushed with 0.2 ml 0.9 % saline. Treated mice received intravenously UK-66P (double dose regimen: 15 mg/kg in a volume of 100 µl immediately and 24 h after bacterial challenge) and control mice received isotype match antibody. Body weight and general appearance was assessed daily during the experiment. Five days post inoculation the mice were euthanized by CO_2 inhalation. Aseptically harvested organs were homogenized in 2 ml saline. Furthermore, the location of the catheter in the superior vena cava was confirmed and the explanted catheter irrigated with 2 ml saline and the irrigation fluid collected. Serial dilutions of the organ homogenates and catheter fluid collections were cultured on mannitol salt phenol red agar plates for at least 48 h at 37℃. The number of bacteria recovered from each organ was plotted versus time postinfection as a Tukey box and whisker plot using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA).

Sepsis survival model

Age and gender matched NMRI mice (Charles River, Sulzfeld, Germany) were used in the experiment. Animals were challenged on day 0 by intravenous injection with 5×10^8 cfu of wild-type S. aureus USA300 and MA12 or IsaA mutant S. aureus MA12 ΔisaA. Treated mice received intravenously UK-66P (double dose regimen: 15 mg/kg in a volume of 100 µl immediately and 24 h after bacterial challenge) and control mice received an isotype match control antibody. Animals were monitored for 8 days, and lethal disease was recorded.

Statistical analysis

Statistical analysis between treated and control groups were performed using the non-parametric Mann-Whitney test. Bacterial burdens in the organs were additionally analyzed by Kruskal-Wallis one-way analysis of variance by ranks with Dunn's posttest. The Log-Rank/Mantel-Cox test was used to analyze the statistical significance of the survival data. For all comparisons, a P value of $< .05$ was considered statistically significant.

RESULTS

Affinity of anti-IsaA monoclonal antibodies

Biosensor analyses of multiple monoclonal antibodies to IsaA were initiated to characterize the binding profiles. Preliminary ELISA studies screened a panel of hybridomas for binding to recombinant IsaA protein (rIsaA). Antibodies that were ELISA positive were applied for a secondary screen. Biacore studies analyzed positive hybridoma clones for high-affinity interaction with rIsaA. Applying the settings as described above, a specific anti-IsaA antibody solution (UK-66P) interacted with the 29 kDa rIsaA antigen with high affinity and slow off-rate indicating a strong and highly specific interaction (Fig. 1). In the experiments with serial antibody dilutions in the range of 0.8 to 400 nM an equilibrium dissociation constant (K_D) of 1.7 nM was determined. Rate constants for association and dissociation of the interaction between UK-66P and rIsaA were determined to be 1.8×10^{5} $M^{-1}s^{-1}$ (k_a) and 3.0×10^{-4} s^{-1} (k_d), respectively.

Functional characterization of UK-66P

The monoclonal antibody UK-66P was selected for further studies based on its high binding affinity to rIsaA. UK-66P was determined to be of IgG1 subclass and indirect immunofluorescence on viable S. aureus showed its ability to bind to the cell surfaces exposed IsaA antigen. Strain MA12, the isogenic IsaAknockout strain MA12 $_{\Delta}$isaA and a protein A knockout strain Cowan I $_{\Delta}$spa were used for staining. Bacteria were incubated with UK-66P followed by fluorescein-labeled anti-mouse Ig and binding of UK-66P to the bacterial surface was visualized by fluorescence microscopy (Fig. 2A-2C). Viable cells of S. aureus MA12 interacted with the IsaA specific monoclonal antibody UK-66P, while the IsaA mutant strain did not interact,

as demonstrated by lack of fluorescence on these cells. The *S. aureus* protein A knock out strain also bound UK-66P indicating no cross-reactivity with protein A.

The targeted IsaA antigen is found conserved among all sequenced methicillin-sensible and methicillin-resistant staphylococcal strains. Subsequently, binding of UK-66P was exemplary tested to a collection of seven representative isolates including strain ANS46 (SCCmec III), strain BK2464 (SCCmec II), strain HDE288 (SCCmec IV), strain MU50 (vancomycin-resistant S. aureus (VRSA)), strain MW2 (CA-MRSA), strain USA300 (CA-MRSA) and strain EMSRA-15 (epidemic MRSA). UK-66P reacted with IsA of all tested strains, as verified by Western blotting analysis (Fig. 2D).

UK-66P mediate activation of professional phagocytes

As the monoclonal antibody UK-66P recognizes IsaA on the surface of *S. aureus*, secondary experiments were performed to determine if the antibody UK-66P could also act as an opsonizing agent resulting in the engulfment of bacteria by activated neutrophils. To quantify phagocytosis, we scored the percentage of oxidizing neutrophils. Neutrophils in whole mouse blood were co-incubated with either wild-type or IsaA mutant *S. aureus* in the presence of UK-66P, isotype control antibody (IC) or saline at 30 and 60 min (Fig. 3A-3B). At both time points, the fraction of oxidizing neutrophils was significantly higher after co-incubation of wild-type bacteria with UK-66P than for the isotype control co-incubated bacteria (Mann-Whitney $P < .05$). More precisely, in the presence of UK-66P (0.6 mg/ml), 46.1% and 97.7% of neutrophils were activated during a co-incubation period of 30 and 60 min, respectively (Fig. 3A). The mean percentage of oxidizing neutrophils was 20.9% and 55.2% with IC after 30 and 60 min incubation time. In the absence of antibodies (saline), the mean percentage of oxidizing neutrophils was 11.8% and 45.5% after

incubation time of 30 min and 60 min. Specificity controls followed the same experimental setup except for using IsaA mutant *S. aureus* (Fig. 3B). Under these conditions, no UK-66P-dependent increase in the percentage of activated neutrophils was detectable when compared to IC and saline primed neutrophils. In conclusion, in the presence of UK-66P (0.6 mg/ml), 8.8% and 56% of neutrophils were activated during a co-incubation period of 30 and 60 min, respectively (Fig. 3B). The mean percentage of oxidizing neutrophils was 24.8% and 66.2% with IC after incubation time of 30 and 60 min. In the control incubated with saline, the mean percentage of oxidizing neutrophils was 21% and 53% after incubation time of 30 and 60 min.

Oxidative burst of neutrophils in response to stimulation with UK-66P

Since increased UK-66P-dependent neutrophil activation does not necessarily imply enhanced bacterial elimination, we measured the production of superoxide quantity per individual neutrophil during phagocytosis of *S. aureus* in professional phagocytes. Neutrophils in whole mouse blood were incubated with UK-66P opsonized wild-type or IsaA mutant *S. aureus*. The production of reactive oxygen metabolites was determined via flow cytometric analysis by measuring the conversion of fluorogenic substrate, DHR 123, to fluorescent R123. Oxidative burst induced by wild-type *S. aureus* in the absence of extrinsic opsonin (saline) was defined as baseline mean fluorescence intensity (MFI, 331). The graph shows that oxidation quantity by wild-type *S. aureus* in the presence of UK-66P at 0.6 mg/ml being significantly higher than that after co-stimulation with IC (MFI, 688 and 366; Mann-Whitney P = .029; Fig. 4A and 4C). Furthermore, the production of superoxide was dose-dependent as half of the UK-66P dose (0.3 mg/ml) did not significantly increase the oxidation quantity compared to IC (MFI, 564 and 366; Mann-Whitney P = .057 Fig. 4A and 4C). The UK-66P-dependent specificity was demonstrated by

failure to produce a significant potentiation of oxidative burst following co-stimulation with the mutant S. aureus strain lacking IsaA expression. The level of oxidative burst was equal to isotype control opsonized or non-opsonized (saline) mutant bacteria, with a MFI of 284 and 287 for UK-66P at dose of 0.3 and 0.6 mg/ml, 294 for IC and 290 for saline (Fig. 4B and 4C). Altogether, the oxidative burst activity per neutrophil stimulated with wild-type S. aureus in the presence of adequate UK-66P was approximately two-fold higher than that of the controls. These results suggest that UK-66P is not only an activator for professional phagocytes but also a stimulus for oxidative burst activity in a dose-dependent fashion.

Effect of UK-66P on the bactericidal activity of professional phagocytes to S. aureus

Using an ex vivo system we determined if the increased oxidant activity of UK-66P translates to enhanced bacterial killing by innate immune mechanism. After the addition of either UK-66P or IC opsonized wild-type and IsaA mutant S. aureus to 100 µl whole mouse blood for 30 min, samples were plated to enumerate survivors after neutrophil lysis. The initial inoculum applied consisted of $5x10^7$ bacteria. Opsonization of bacteria with UK-66P significantly enhances bacterial killing by whole blood neutrophils compared to IC opsonized bacteria (mean cfu ± SD, $1.13x10^5 \pm 9.38x10^3$ and $2.99x10^5 \pm 3.65x10^3$; Mann-Whitney P = .0286; Fig. 5). This effect was not explainable by differences in the phagocytosis rate, since uptake of the wild-type S. aureus was fairly comparable in the presence of either UK-66P or IC antibodies (data not shown). Therefore, differences in phagocytotic killing were clearly attributable to binding of UK-66P to IsaA, since UK-66P and IC treated IsaA mutant S. aureus produced similar results (mean cfu ± SD, $1.8x10^5 \pm 1.3x10^4$ and $1.9x10^5 \pm 6.1x10^3$; Fig. 5).

Therapeutic efficacy of UK-66P in a catheter-related sepsis model

In order to stress the importance of the ex vivo results we tested the therapeutic efficacy of UK-66P in a sublethal catheter-related sepsis model in mice. Bacterial challenge and treatment was executed 24h after microsurgical implantation of a catheter into the internal jugular vein of mice. Animals with weight loss greater than 5% after surgery compared to baseline values were excluded from the study to avoid compounding effects of anaesthesia or surgical preparation. All elected mice were challenged with $1x10^{7}$ cfu *S. aureus* MA12 via the catheter. Mice were then treated via the catheter with UK-66P (N = 7; double dose regimen: 15 mg/kg in a volume of 100 µl immediately and 24 h after bacterial challenge to yield an effective dose of 30 mg/kg) or IC (N = 9; dose and volume equivalent). Mice were sacrificed five days after *S. aureus* challenge. Numbers of viable bacteria in the liver, lung, heart, spleen and kidneys following therapy with either UK-66P or IC were quantitated and graphed against time postinfection (Fig. 6). In addition, the catheters were collected and indwelling bacteria obtained. Isotype control antibody treated mice preferentially colonized the kidneys over the infection period. Analysis of the bacterial load in this organ system revealed a significant difference in the numbers of recovered wild-type *S. aureus* after UK-66P or IC treatment ($P < .05$; Kruskal-Wallis test with Dunn's posttest), with a greater recovery of bacteria in IC-treated animals. The protective effect of the UK-66P treatment was approximately three logs. In contrast, the lung, liver, heart and spleen cfu did not differ between UK-66P and IC treated mice. The specific infection burden within each organ system is presented in table 1. In addition, the amount of bacteria obtained from the catheters in the IC group was not significantly different compared to the amount of bacteria from

412 catheters in the UK-66P treatment group (mean cfu (range), 2.8x10 ^{5} ($1.0x10^{5}$ -
413 $3.1x10^{6}$) and $1.3x10^{6}$ ($1.3x10^{5}$ - $2.3x10^{6}$)).
414
415 **UK-66P treatment protects animals against lethal challenge with S. aureus**
416 In order, to corroborate the obtained results, a different experimental model
417 was introduced. Effective immunotherapy to S. aureus should protect mice against a
418 lethal challenge of S. aureus. Furthermore, immunotherapy must be effective against
419 a wide range of clinically relevant isolates. Therefore, S. aureus strain USA300 was
420 additionally included. USA300 is one of the most frequent cause of community-
421 associated infections in the United States (26), and protection against this strain is of
422 crucial importance for immunotherapeutic efforts. To test whether UK-66P
423 immunotherapy protects against lethal-challenge infections, mice were treated
424 intravenously with UK-66P or isotype control antibody. Challenges of 5×10 ^{8} cfu of
425 wild-type S. aureus USA300, MA12 and IsaA mutant (MA12 ΔisaA) were
426 administered intravenously, and mice were monitored for 8 days. Isotype control
427 antibody treatment in wild-type S. aureus USA300 and MA12 had no effect with 57%
428 and 75% mortality rate, respectively over study period (Fig. 7). In contrast, UK-66P
429 treatment protected against challenge with S. aureus USA300 (0% mortality rate;
430 Log-Rank/Mantel-Cox P = .038) and S. aureus MA12 (25% mortality rate; Log-
431 Rank/Mantel-Cox P = .041). Clearly, the immunotherapeutic potential of UK-66P was
432 demonstrated by the fact that mice challenged with S. aureus MA12 ΔisaA were not
433 protected by anti IsaA antibodies compared to isotype control treated mice (50 and
434 38% mortality rate; Log-Rank/Mantel-Cox P = .55). These results suggest that
435 passive immunotherapy with UK-66P monoclonal antibodies can generate increased
436 protection against lethal challenge.
437

DISCUSSION

S. aureus causes a wide variety of diseases ranging from superficial skin lesions to life-threatening invasive infections and those at high-risk include individuals with short-term or permanent states of immunosuppression, such as patients undergoing surgery, cancer patients, ICU patients, HIV patients and patients with dialysis dependence, diabetes or HIV (28, 30). There is still a high mortality rate associated with severe S. aureus infections especially those caused by multiple antibiotic-resistant strains. Therefore, active or passive immunotherapy has been regarded as a promising adjunctive treatment approach that can bolster the immune response and circumvent rising rates of antimicrobial drug resistance. Experimental evidence strongly supports the concept but successful clinical trials are still pending (35).

In the present report, the therapeutic efficacy of the mouse monoclonal antibody UK-66P binding the immunodominant antigen IsaA has been evaluated in vivo. The UK-66P hyperimmune preparation, passively applied in two different experimental mouse models, lowered significantly S. aureus burden in kidneys and protects mice against lethal challenge. The kidneys are the predominant infection site in mice after intravenous challenge with S. aureus (32, 39). Lowering the bacterial load in this single organ system limit the establishment of infectious foci and thereby curb the severity of staphylococcal infections. The specific IgG1 subclass preparation was chosen for the experimental studies, as secondary IgG1 immune response seems to be crucial in S. aureus infections (16, 29). High affinity binding kinetics of antibodies was demonstrated by Biacore analysis.

Further investigation was conducted to clarify the UK-66P antibody mode of action. Obviously, UK-66P has not a direct blocking function to IsaA. Instead, binding of UK-66P to IsaA on the cell surface triggers phagocytosis and subsequent

464 intracellular killing of S. aureus. The bactericidal activity of neutrophils after UK-66P
465 treatment correlates with augmentation in the respiratory burst of these cells The
466 observation of neutrophil killing subjected to high concentrations of superoxide and
467 hydrogen peroxide is concordant with other studies (13). Likewise, mice lacking
468 NADPH oxidase activity clear inefficiently S. aureus infections (33). The ex vivo
469 results of the antibody mediated phagocytosis transferred to the catheter related
470 infection and sepsis model may explain the effective reduction of S. aureus in the
471 treated hosts with improved survival. Phagocytosis and intracellular killing of S.
472 aureus will decrease the initial bacterial proportion helping to displace the infective
473 balance towards the host organism (1). In addition, the percentage of surviving S.
474 aureus inside various cells including phagocytes contributes to the pathogenesis of
475 staphylococcal infections (11). In our ex vivo studies, the UK-66P immunotherapy
476 reduced the amount of viable S. aureus within phagocytes by one-third compared
477 with the control. In this context, a recent study examined the comparative lethality of
478 clinical S. aureus isolates in a mouse septic model and lowering the infectious dose
479 of viable bacteria by approximately one-third led to a marked decrease in mortality
480 within a given time (40).

481 Originally, IsaA has been identified in a screening approach for
482 immunodominant antigens that may serve as vaccine candidates or targets for
483 passive immunotherapy (24). The surface-associated IsaA antigen is found
484 conserved among all sequenced staphylococcal strains including community-
485 associated methicillin-resistant S. aureus such as USA300 (41). Moreover, we
486 demonstrated binding of UK-66P to IsaA of major clinical S. aureus lineages. In vivo
487 data suggest that antibody reactivity raised to IsaA tends to be a ubiquitous
488 mechanism in host immune defence to invasive S. aureus infections (9, 24).
489 Importantly, reactive IgG titers against IsaA are significantly increased in serum

samples obtained from individuals with confirmed disease compared to those from healthy individuals. Furthermore, comparison of IgG titers against IsaA between healthy non-carriers and carriers showed that there are significantly increased reactive titers in the later group (6). We hypothesize that the amount of anti-IsaA specific antibodies represents one important component that modulate the outcome of human systemic S. aureus infection. Nevertheless, the significance of the antibody response to IsaA in a human infection has not yet been conclusively defined.

Selection of a protective target antigen is the key factor in the development of an effective vaccine or passive immunization strategy. Traditionally, virulence associated factors are regarded as promising vaccine candidates due to their potential role in pathogenesis. This concept is especially in S. aureus questionable since the pathogen expresses a broad range of toxins and adhesins which are important in different phases of disease but are not solely essential for virulence as others may compensate a loss of function e.g. by blocking antibodies. Alternatively, natural raised antibodies of humans exposed to staphylococcal invasive infections may select antigens as potential targets for immunotherapy (6, 24, 27, 46). However, as native protective immunity to staphylococcal infections does not exist at a significant degree and individuals with colonization to invasive infections elicit a unique immune response to different S. aureus proteins, selection of an universal protective antigen is highly challenging (14, 42). Moreover, certain patients develop recurrent infections, usually with the same strain in up to 35% despite a broad spectrum of existing antistaphylococcal antibodies (15). On the other hand, those patients that become infected with their own strain have a significant higher chance to survive invasive S. aureus infections (45).

Although our approach has demonstrated to be effective in two animal model systems clinical trials are still lacking. Very recently, we have identified the antigen

binding region of UK66-P and grafted the mouse complementarity determining regions into human variable regions which were joined to human constant regions. Studies on binding and functional activity of the humanized antibody are currently in progress. However, some important issues remain to be addressed before this antibody may proceed to clinical trials. For example, the optimal treatment regimen has to be determined as well as the general accessibility of IsaA in all strains including those of different capsule types.

Together, the results presented in this study have demonstrated that the immunotherapy strategy to IsaA targeting only a single epitope of S. aureus is effective in reducing bacteria in experimental infections due to enhanced opsonophagocytosis with effective intracellular killing. Based on the mechanism of action, the ability of therapeutic antibodies to work cooperatively with the immune system in this way has important implications for the selection of IsaA as target for immunotherapy of staphylococcal infections. Further analysis of the humanized antibody will comply with this expectation.

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LEGENDS OF FIGURES

FIG. 1. Quantitative analysis of the interaction of UK-66P to rIsaA using surface plasmon resonance. Various concentrations of rIsaA (0.8 - 400 nM) were flushed over the antibody UK-66P, immobilized on the sensor chip surface. Sensorgrams were recorded at a flow rate of 30 µl/min at 25℃. From these sensorgrams an equilibrium dissociation constant (K_D) of 1.7 nM was determined. Rate constants for association and dissociation were determined to be $1.8x10^5$ $M^{-1}s^{-1}$ (k_a) and $3.0x10^{-4}$ s^{-1} (k_d), respectively. The figure shows one representative result of two independent experiments yielding identical kinetic constants.

FIG. 2. Display of UK-66P on the staphylococcal surface and positive binding to representative clinical *S. aureus* isolates are shown. Binding of FITC labeled anti-mouse IgG to UK-66P was analyzed by conventional microscopy of *S. aureus* (upper panel) and corresponding fluorescent microscopy with superimposing the data (lower panel). (A) UK-66P binds specifically wild-type *S. aureus* MA12. (B) The isogenic mutant strain MA12 Δisa failed to bind UK-66P. (C) *S. aureus* protein A knock out strain (Cowan I Δspa) binds UK-66P indicating no antibody cross reactivity with protein A (x 100 magnification). (D) Reactivity of UK-66P to IsaA of representative clinical isolates including strain ANS46 (SCCmec III), strain BK2464 (SCCmec II), strain HDE288 (SCCmec IV), strain MU50 (vancomycin-resistant S. aureus (VRSA)), strain MW2 (CA-MRSA), strain USA300 (CA-MRSA) and strain EMSRA-15 (epidemic MRSA) was constant, as verified on western blotting

FIG. 3. UK-66P activates neutrophils. 100 µl mouse blood were incubated with $5x10^7$ cfu of either UK-66P opsonised wild-type *S. aureus* MA12 or IsaA mutant *S. aureus* MA12 ΔisaA. Controls included isotype control antibody (IC) opsonized and

771 unopsonized (saline) bacteria. The percentages of activated and oxidizing
772 neutrophils were determined using a DHR123/R123 assay in flow cytometric
773 analysis. (A) At 30 and 60 min, the fraction of oxidizing neutrophils was significantly
774 higher in the presence of wild-type bacteria with UK-66P than for the IC and saline
775 co-incubated bacteria (Mann-Whitney $P < .05$). (B) As a specificity control, the
776 respective percentages of oxidizing neutrophils were similar after UK-66P, IC or
777 saline co-incubated IsaA mutant bacteria.
778
779 **FIG. 4.** Oxidative burst of neutrophils is significantly enhanced in response to UK-66P
780 opsonized S. aureus. The oxidative burst activity of native mouse blood neutrophils
781 was determined using a DHR123/R123 assay and flow cytometric analysis. (A)
782 Oxidative burst was monitored by observing the fluorescence events (M1) in FL1
783 overlay histogram. Wild-type S. aureus MA12 stimulated neutrophils with the addition
784 of saline (black line), isotype control antibody (IC, grey line) or UK-66P at a
785 concentration of 0.3 mg/ml (red line) and 0.6 mg/ml (blue line). (B) As a specificity
786 control for UK-66P, the oxidative burst was additionally monitored for IsaA mutant S.
787 aureus MA12 ΔisaA stimulated neutrophils with the addition of saline (black line), IC
788 (grey line) or UK-66P at a concentration of 0.3 mg/ml (red line) and 0.6 mg/ml (blue
789 line). (C) Mean of the fluorescence intensity (MFI) of UK-66P opsonized bacteria at a
790 concentration of 0.3 and 0.6 mg/ml (body weight equivalent of 15 mg/kg and 30
791 mg/kg) compared to IC or saline opsonized wild-type and IsaA mutant strain.
792 Significant differences are denoted (Mann-Whitney test).
793
794 **FIG. 5.** Effect of UK-66P on survival of S. aureus within neutrophils in whole mouse
795 blood. 100 µl mouse blood were incubated with $5x10^7$ cfu of either UK-66P opsonised
796 wild-type S. aureus MA12 or IsaA mutant S. aureus MA12 ΔisaA. Controls included

isotype control antibody (IC) opsonised bacteria. The number of neutrophil-associated cfu were determined by serial dilution and plating on TSB. UK-66P opsonized wild-type S. aureus were killed significantly better than the IC opsonized bacteria (mean cfu ± SD, $1.13x10^{5} \pm 9.38x10^{3}$ and $2.99x10^{5} \pm 3.65x10^{3}$; Mann-Whitney P = .0286). UK-66P and IC opsonized IsaA mutant S. aureus produced similar results (mean cfu ± SD, $1.8x10^{5} \pm 1.3x10^{4}$ and $1.9x10^{5} \pm 6.1x10^{3}$).

FIG. 6. Bacterial burden after UK-66P and isotype control antibody treatment in lung, heart, liver, spleen and kidneys five days after infection with S. aureus MA12 in the catheter-related infection model. The mice (N = 7-9 per group) were inoculated via the catheter with $1x10^{7}$ cfu bacteria. * P < .05 compared with IC treated mice by Kruskal-Wallis testing with post-hoc Dunn's multiple comparison testing. Data are graphed as Tukey box and whisker plot.

FIG. 7. Immunotherapy with UK-66P generates protection against lethal S. aureus challenge. Mice (N = 7 - 8 per group) were given UK-66P antibody preparation or isotype control antibody (IC). Animals were challenged with 5×10^{8} cfu of wild-type S. aureus USA300, MA12 or IsaA mutant S. aureus MA12 ΔisaA by intravenous injection and then they were monitored for 8 days. Compared with animals receiving control IgG1, the significance of protection was measured with the Log-Rank/Mantel-Cox Test.

823 **TABLE 1** Multiorgan infection caused by S. aureus after UK-66P and isotype control
824 antibody treatment in the catheter-related sepsis model.
825

Organ	**S. aureus** MA12 + isotype control mean (range)	**S. aureus** MA12 + UK-66P mean (range)	p
Kidneys	$1.1x10^6$ ($7.5x10^1$ - $1.4x10^8$)	$4.0x10^2$ (0 - $4.2x10^7$)	< .05*
Lung	$1.3x10^3$ (0 - $8.6x10^7$)	$1.4x10^2$ (0 - $1.8x10^4$)	n.s.
Liver	$2.0x10^3$ ($4.0x10^1$ - $1.1x10^8$)	$2.8x10^2$ (0 - $4.0x10^4$)	n.s.
Spleen	$1.6x10^2$ (0 - $5.1x10^5$)	$2.0x10^1$ (0 - $2.9x10^3$)	n.s.
Heart	$1.8x10^2$ (0 - $3.6x10^7$)	$1.8x10^2$ (0 - $3.3x10^5$)	n.s.
		$1.1x10^3$ ($1.0x10^2$ - $5.5x10^5$)	

826 * Kruskal-Wallis test with Dunn`s posttest; n.s. not significant

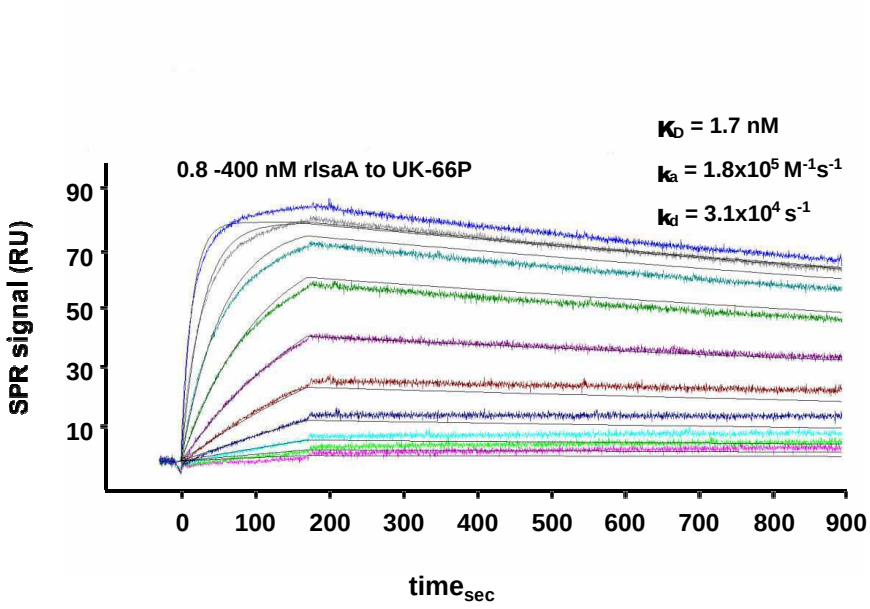
K_D = 1.7 nM
k_a = 1.8x10^5 M^{-1}s^{-1}
k_d = 3.1x10^4 s^{-1}
0.8 -400 nM rIsaA to UK-66P
SPR signal (RU)
90
70
50
30
10
0
100
200
300
400
500
600
700
800
900
time$_{sec}$

Fig. 1

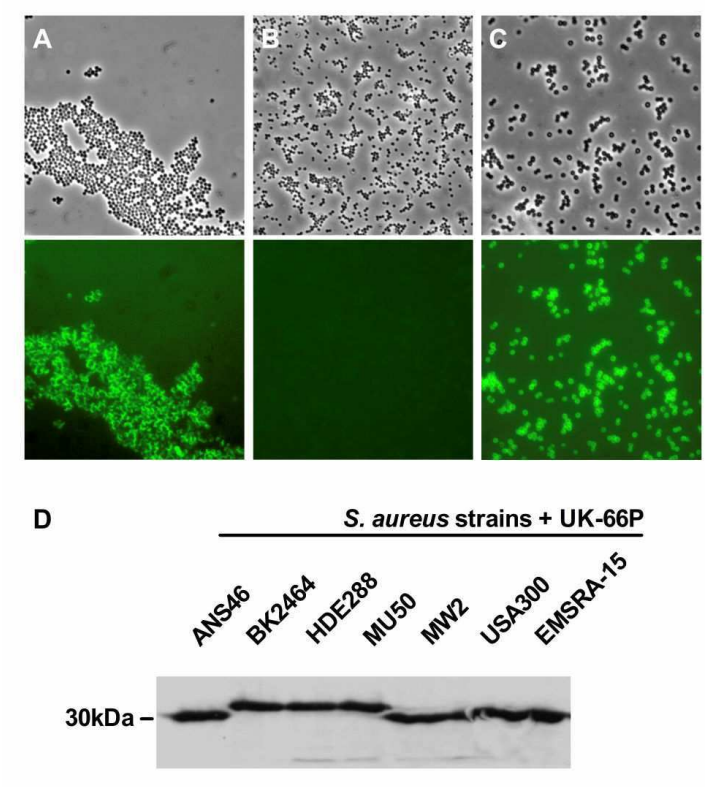
A
B
C
D
S. aureus strains + UK-66P
ANS46
BK2464
HDE288
MU50
MW2
USA300
EMSRA-15
30kDa –

Fig. 2

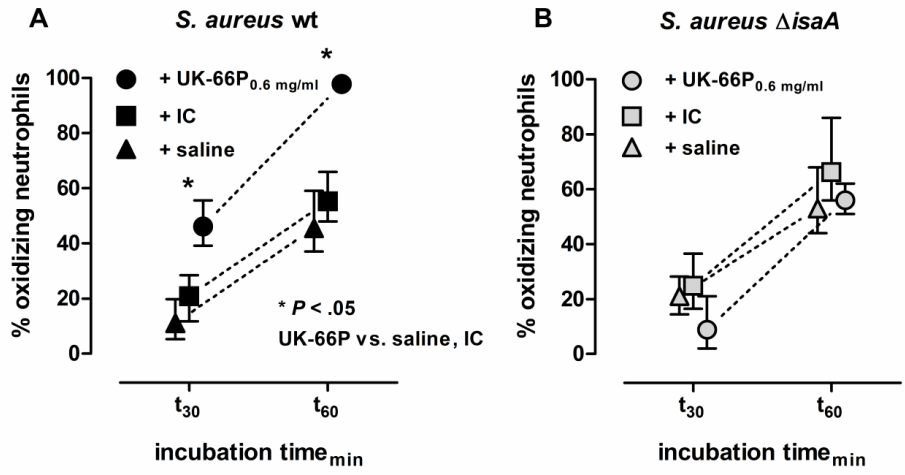
A
S. aureus wt
% oxidizing neutrophils
100
80
60
40
20
0
+ UK-66P0.6 mg/ml
+ IC
+ saline
*
*
* P < .05
UK-66P vs. saline, IC
t30
t60
incubation timemin
B
S. aureus ΔisaA
% oxidizing neutrophils
100
80
60
40
20
0
+ UK-66P0.6 mg/ml
+ IC
+ saline
t30
t60
incubation timemin

Fig. 3

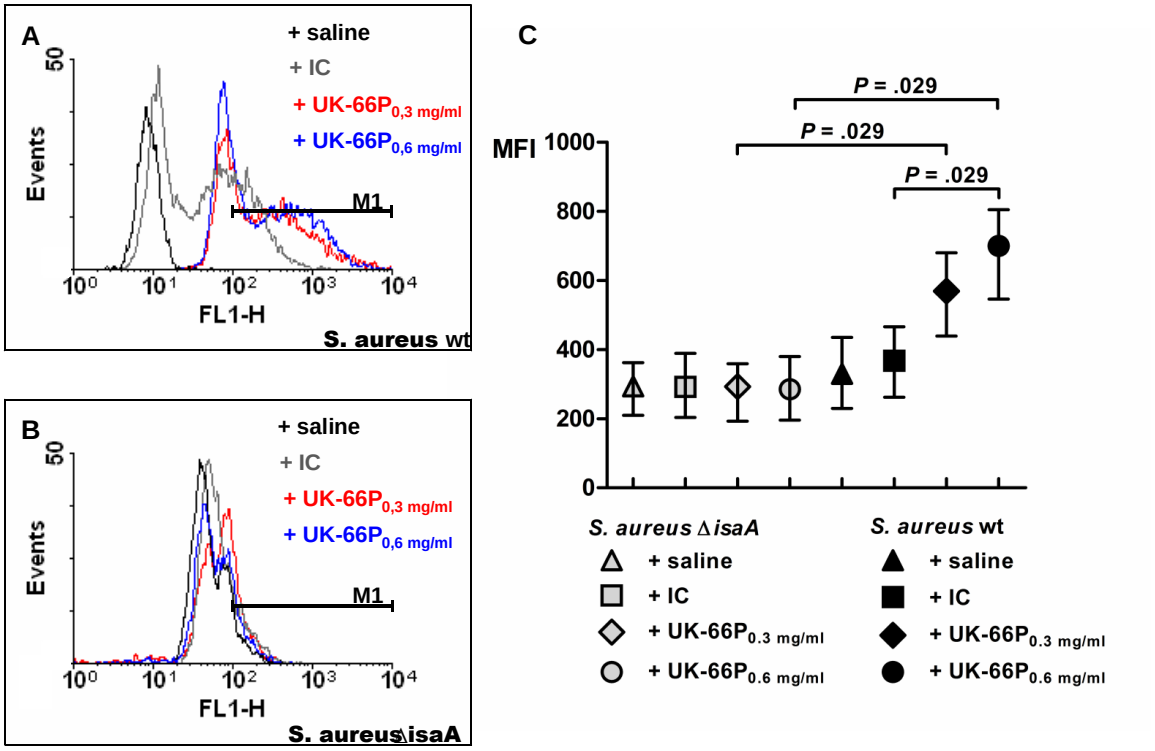
A
+ saline
+ IC
+ UK-66P0,3 mg/ml
+ UK-66P0,6 mg/ml
Events
50
M1
10⁰ 10¹ 10² 10³ 10⁴
FL1-H
S. aureus wt
B
+ saline
+ IC
+ UK-66P0,3 mg/ml
+ UK-66P0,6 mg/ml
Events
50
M1
10⁰ 10¹ 10² 10³ 10⁴
FL1-H
S. aureus ΔisaA
C
P = .029
P = .029
P = .029
MFI
1000
800
600
400
200
0
S. aureus ΔisaA
+ saline
+ IC
+ UK-66P0.3 mg/ml
+ UK-66P0.6 mg/ml
S. aureus wt
+ saline
+ IC
+ UK-66P0.3 mg/ml
+ UK-66P0.6 mg/ml

Fig. 4

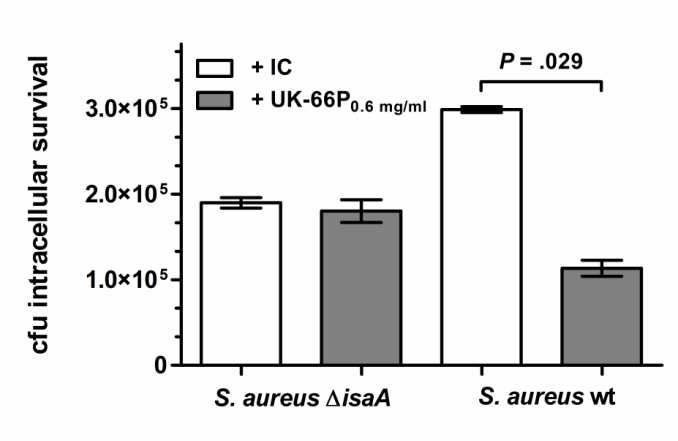
cfu intracellular survival
+ IC
+ UK-66P0.6 mg/ml
P = .029
3.0×10^5
2.0×10^5
1.0×10^5
0
S. aureus ΔisaA
S. aureus wt

Fig. 5

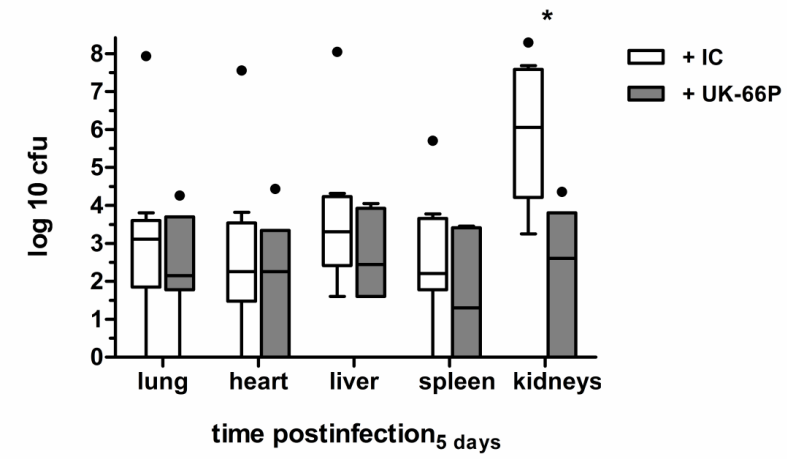
log 10 cfu
0
1
2
3
4
5
6
7
8
lung
heart
liver
spleen
kidneys
*
+ IC
+ UK-66P
time postinfection5 days

Fig. 6

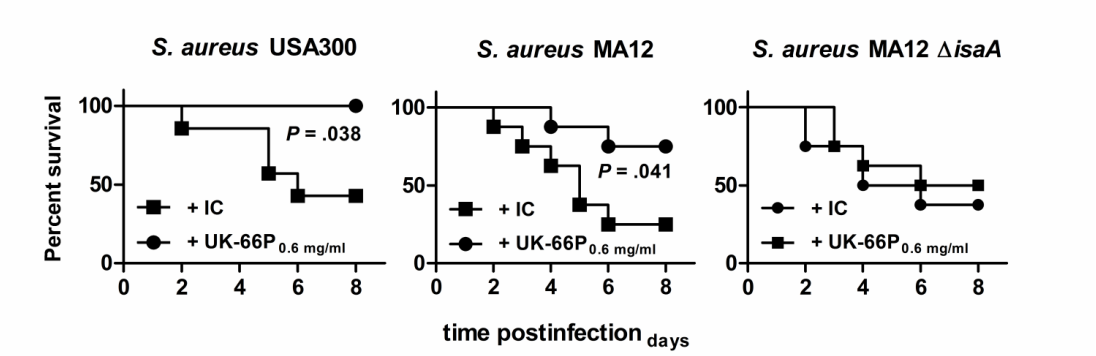
S. aureus USA300
S. aureus MA12
S. aureus MA12 ΔisaA
Percent survival
100
50
0
P = .038
P = .041
+ IC
+ UK-66P 0.6 mg/ml
0
2
4
6
8
time postinfection days

Fig. 7