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

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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 (Lowy, 1998; Sibbald et al., 2006). 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 (Cosgrove, 2006; Cosgrove et al., 2005). In recent years, a spread of resistant staphylococci has also been observed outside health care settings, due to community-acquired MRSA (Chambers and DeLeo, 2009). 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 (Chambers and DeLeo, 2009), there is a critical need for alternative antimicrobial intervention strategies.

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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 (Verkaik et al., 2011). 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 (Ziebandt et al., 2010). Notably, IsaA is a non-covalently cell wall-bound lytic transglycosylase (Lorenz et al., 2000; Sakata et al., 2005; Stapleton et al., 2007), which is co-regulated with the glycylglycine endopeptidase LytM (Dubrac and Msadek, 2004). 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 (Dreisbach et al., 2010). In humans, IsaA is highly immunogenic, and *S. aureus*-infected patients have increased anti-IsaA IgG levels (Clarke et al., 2006; den Reijer et al., 2013; Lorenz et al., 2000; van der Kooi-Pol et al., 2013). 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. (Lorenz et al., 2011) 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 (Ziebandt et al., 2010). Recombinant strains lacking IgG-binding proteins included *S. aureus* Newman Δ*spa* Δ*sbi* (Sibbald et al., 2010) and SH1000 Δ*spa* (Sibbald et al., 2012). 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, Corsham, England) as described earlier (van der Kooi-Pol et al., 2013). *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, Veenendaal, The Netherlands).

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, Darmstadt, Germany), resulting in plasmid pET24dIsaA. For IsaA production, an overnight culture of *E. coli* BL21DE3 (pET24dIsaA) was diluted 1:100 in fresh Lysogeny Broth (BD, Breda, The Netherlands). Four h 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, Etten-Leur, The Netherlands) chilled on ice. Cleared lysate was mixed with His Mag Sepharose™ Ni magnetic beads (GE Healthcare, Diegem, Belgium) 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 (Sibbald et al., 2010). Protein samples were separated using NuPAGE gels (Life Technologies, Bleiswijk, The Netherlands) according to the manufacturer's instructions and proteins were transferred to a Protran nitrocellulose membrane (Whatman, 's-Hertogenbosch, The Netherlands) by semi-dry blotting (75 min at 1 mA/cm^2). Membranes were incubated with specific rabbit antibodies against IsaA (kindly provided by Sakata (Sakata et al., 2005)) 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, Bad Homburg, Germany).

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). Human peripheral blood cells were separated by Ficoll centrifugation and B lymphocytes were isolated using the EasySep Human B cell enrichment procedure (Stemcell technologies, Grenoble, France). Next, IsaA-specific memory B cells were enriched on a MoFlo cell sorter (DakoCytomation, Glostrup, Denmark) by selecting for biotinylated recombinant IsaA, CD27 and CD19. Selected B cells were incubated on CD40L-expressing 3T6-cells, similar as described in patent US 787415 B2 (Groen and Westra, 2010) in the presence of IL-2 and IL-21, and after 7 days the supernatant was tested for antibodies against IsaA. The human monoclonal IQNPA (Albrecht et al., 2007) was used as isotype control mAb. Positive wells were transferred on ELISPOT plates for 2 days. Cells were transferred back to 96-well plates in the presence of MegaCD40L (Enzo Life Sciences, Antwerpen, Belgium), IL-2 and IL-21, and

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ELISPOT plates were analyzed for spots of anti-IsaA B lymphocytes. Finally, cells from ELISPOT-positive wells were single cell sorted and monoclonal antibodies (IgG1) were cloned after single-cell PCR and expressed with HEK293 cells as described (Smith et al., 2009; Tiller et al., 2008) with minor modifications. We purified mAbs using protein A agarose beads and finally tested them for binding to rIsaA by ELISA as described below.

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 h 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 h at 37 °C. Between each step, the wells were washed 3 times with PBST. Reaction was visualized with TMB substrate (Kem-en-Tec Diagnostics, Taastrup, Denmark) 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 1× (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 5×10^6 CFU per well in PBS for 18 h 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, West Grove, PA, USA) 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, Heverlee, Belgium) and visualized with TMB substrate as above.

Animals

Specified pathogen-free female BALB/cBYJ mice were obtained from Charles River (Saint-Germain-sur-l'Arbresle, France). 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, Cambridge, UK), 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 h at 37 °C in a BioScreen C growth analyzer (Oy Growth Curves Ab Ltd, Helsinki, Finland). OD_{600} was measured every 15 min.

Isolation of neutrophils

Human neutrophils were isolated from heparinized blood of healthy volunteers as described (Troelstra et al., 1997). 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 min at 1500 × *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 h 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, Vilvoorde, Belgium). The reaction was initiated by the addition of 50 μL neutrophils in HBSS/HSA at 1.25×10^6 cells/mL for human and 5×10^6 cells/mL for murine neutrophils, respectively. Oxidative burst was continuously recorded for 30 min 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 h at 4 °C, washed with RPMI/HSA and stored at −20 °C. Phagocytosis with human or mouse neutrophils was performed as described (Stemerding et al., 2013), but extended to 30 min 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 h after inoculation. CFUs 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 (van den Berg et al., 2013), using inocula of $2–4 \times 10^5$ CFU for *S. aureus* isolate P or $4–7 \times 10^5$ CFU for *S. aureus* USA300. Three h before or

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after induction of bacteremia, 1D9 was administered. Discomfort and animal survival rate over 14 days after infection was monitored as described before (van den Berg et al., 2013).

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 h after infection ($n = 4$ per time point) as described (van den Berg et al., 2013).

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 h 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 h after infection and collected in a Microvette® CB300 capillary tube (Sarstedt, Etten-Leur, The Netherlands). 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, Armonk, NY, USA). 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 h 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., La Jolla, CA, USA). 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 (Fig. 1A). Of the latter group, donor T7-1 also tested positive for PA, which was used as a control (Fig. 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 (Fig. 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 (Fig. 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* Newman*Δspa Δsbi* was included. 1D9 bound concentration-dependent to *S. aureus* isolate P, USA300, Newman wild-type and Newman*Δspa Δsbi* (Fig. 2A). Binding of control mAb IQNPA to *S. aureus* 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 (Fig. 2B). In addition, 1D9 bound to 9 sequenced and 16 defined clinical *S. aureus* isolates, including both MSSA and MRSA isolates (Fig. 2C). Binding of IQNPA (100 ng/mL) was relatively low for most strains (Fig. 2D).

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 (Fig. 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.

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 (Fig. 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.

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 Fig. 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 h 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 (Fig. 5). In both infections, ~99% of the staphylococci had disappeared from blood already after 1 h. The bacterial load in spleen and liver decreased within the first 24 h 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 h 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.

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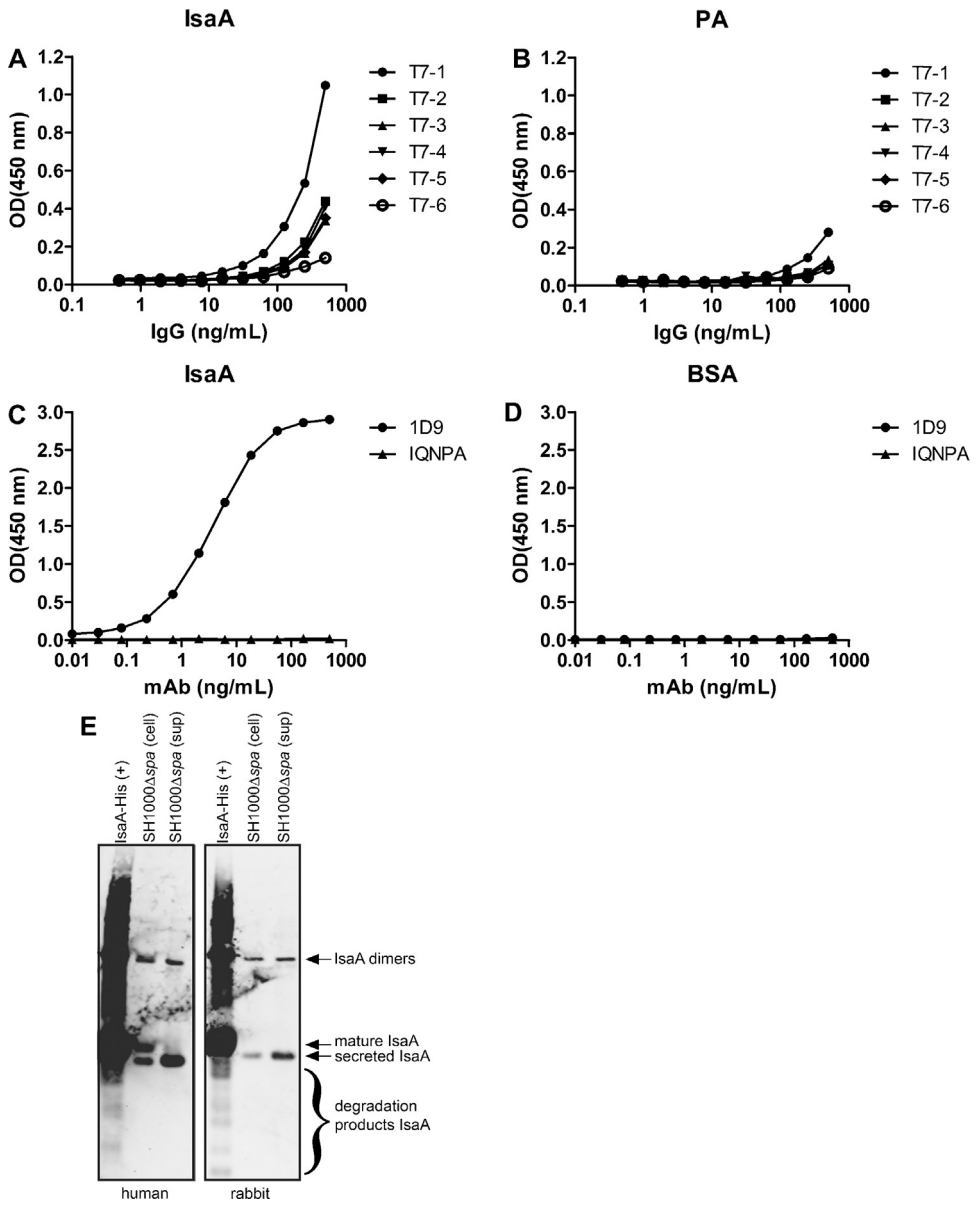

Fig. 1. Donor selection and generation of human monoclonal antibody 1D9 specific for IsaA and *S. aureus*. (A, B) Serum IgG levels against IsaA-His_6 or PA in 6 nasal *S. aureus* carriers. (C, D) Concentration-dependent binding of 1D9 and isotype control antibody (IQNPA) to IsaA-His_6 or BSA. (E) Binding 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.

Course of serum 1D9 levels over time

Mice were treated i.v. with 5 mg/kg 1D9. Three h later, bacteremia was induced by either *S. aureus* isolate P or USA300. Antibody levels at 1, 6, and 24 h were assessed (Fig. 6). In uninfected mice, serum 1D9 levels remained stable during 24 h. 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 h. No differences in 1D9 titers were observed between mice with either *S. aureus* isolate P or USA300 bacteremia.

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 ($P = 0.0057$; Fig. 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 ($P > 0.05$; Fig. 7C). Therapeutic treatment with 5 mg/kg 1D9 did not improve the survival rate of mice with *S. aureus* isolate P or USA300 bacteremia (Fig. 7B and D).

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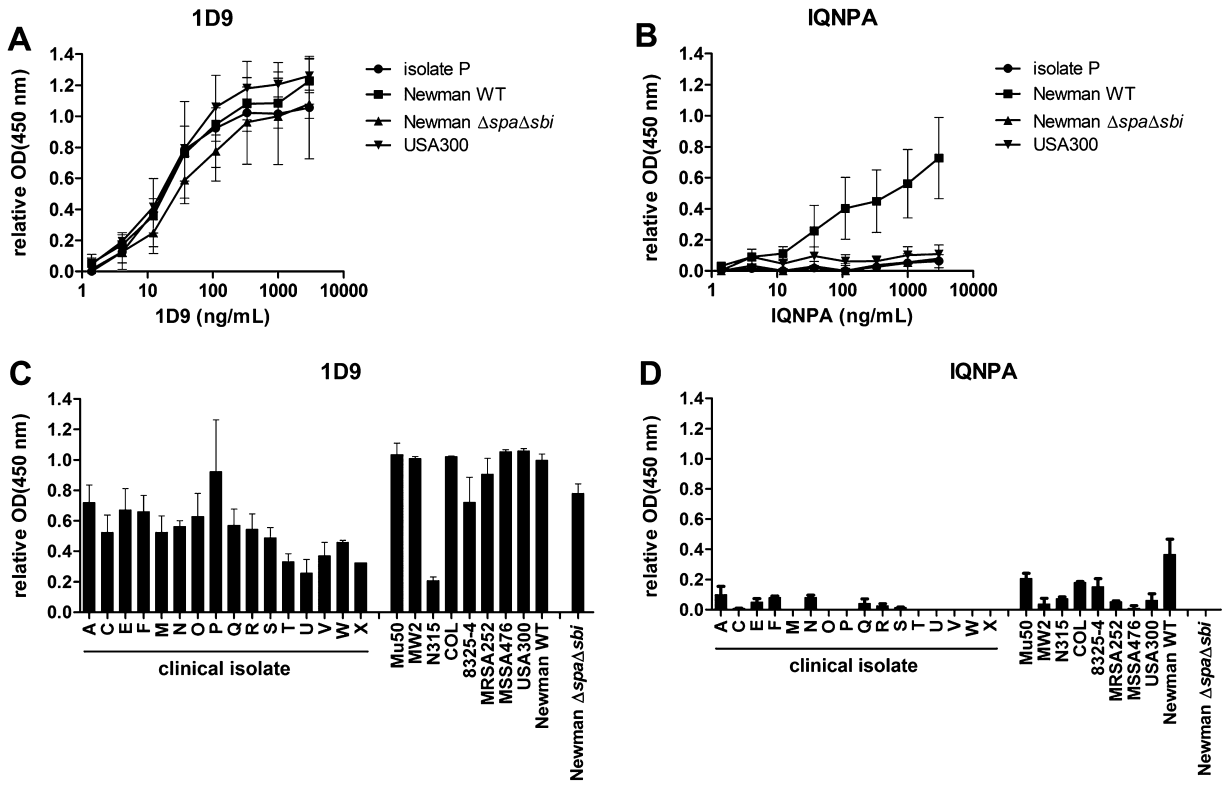

Fig. 2. Binding of 1D9 to *S. aureus* in whole cell ELISA assay. Plates were coated with cells of various *S. aureus* strains harvested at mid-exponential growth. Biotinylated 1D9 or the isotype control antibody IQNPA was added. Absorption values (at 450 nm) are plotted relative to binding of 1000 ng/mL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

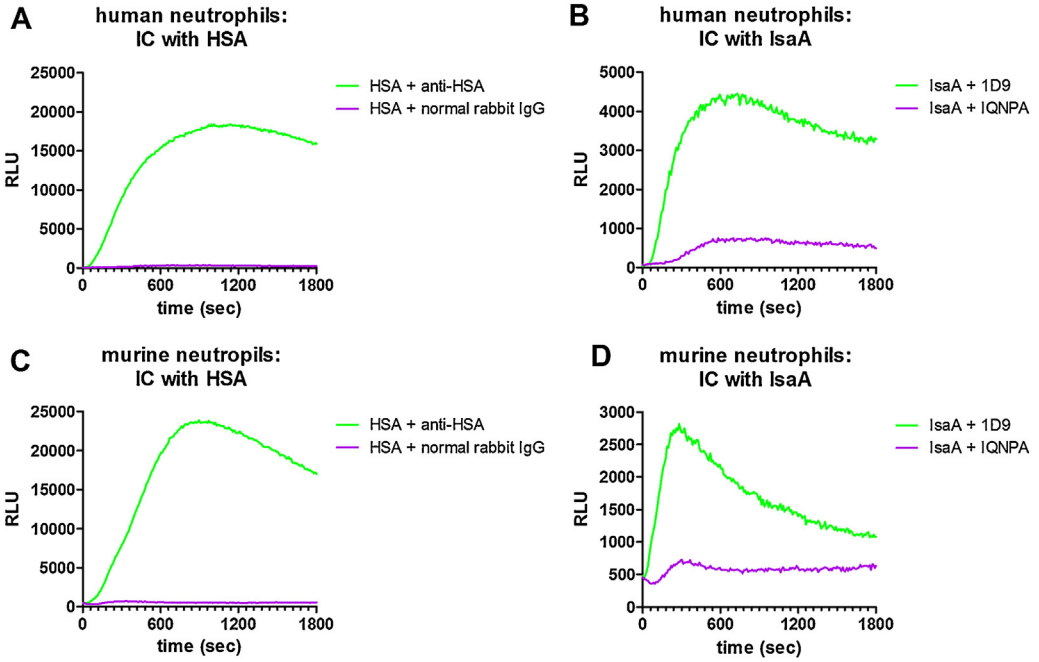

Fig. 3. Activation of human or murine neutrophils by 1D9. Plates were coated with 5 μg/mL HSA (A, C) or IsaA (B, D), blocked with 10% fetal calf serum and incubated with 1 or 3 μg/mL rabbit anti-HSA or 1D9, respectively. Control antibodies were normal rabbit IgG or human mAb IQNPA. Neutrophils (human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

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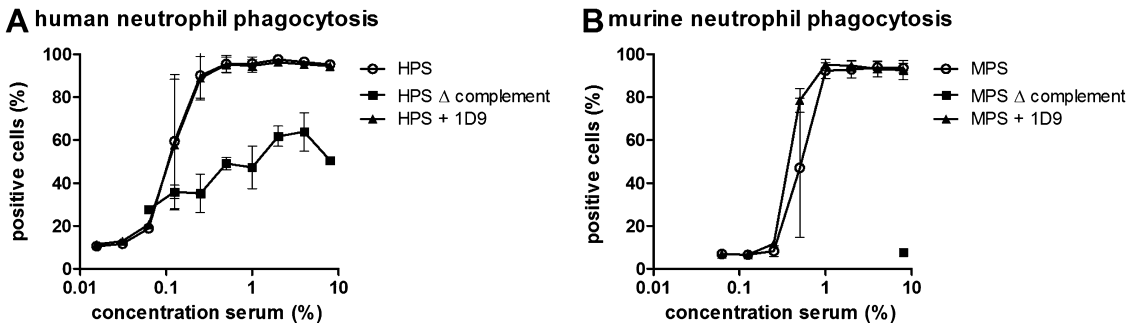

Fig. 4. Phagocytosis of *S. aureus* isolate P by human (A) and mouse (B) neutrophils. Serial dilutions of normal human pooled serum (HPS) or normal mouse pooled serum (MPS), with or without (Δ) complement (inactivated by heating), were mixed with 1D9 (10 μg/mL). *S. aureus* isolate P, labeled with FITC, 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) min 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.

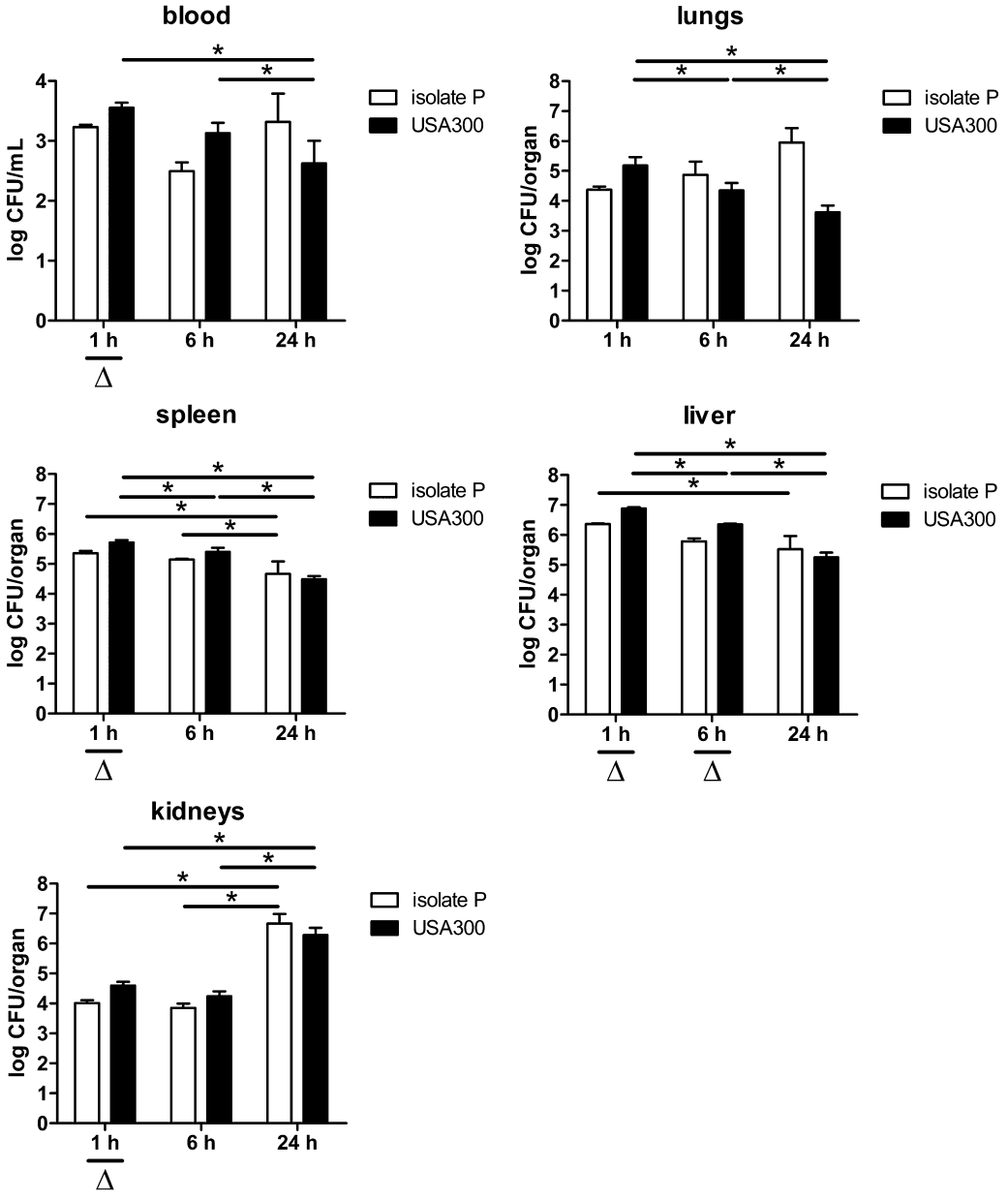

Fig. 5. *S. aureus* counts in blood and organs from mice with *S. aureus* bacteremia. Mice (4 per group) were infected with 2–4 × 10^5 CFU of *S. aureus* isolate P (open bars) or with 4–7 × 10^5 CFU of *S. aureus* USA300 (black bars), by intravenous inoculation, and were sacrificed at indicated time 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, $P < 0.003$).

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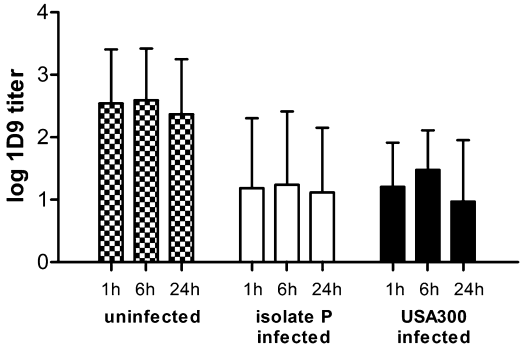

Fig. 6. Course of serum 1D9 levels over time. Mice (3 per group) were treated intravenously with 5 mg/kg 1D9. Three hours later, bacteremia was induced by either *S. aureus* isolate P or *S. aureus* USA300. Uninfected mice were included as well. At indicated time points after infection, serum levels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

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 (Smith et al., 2009; Tiller et al., 2008), 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 (Ziebandt et al., 2010), 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 h 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 (Kelly-Quintos et al., 2006). They used mAb concentrations ranging from 25 to 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

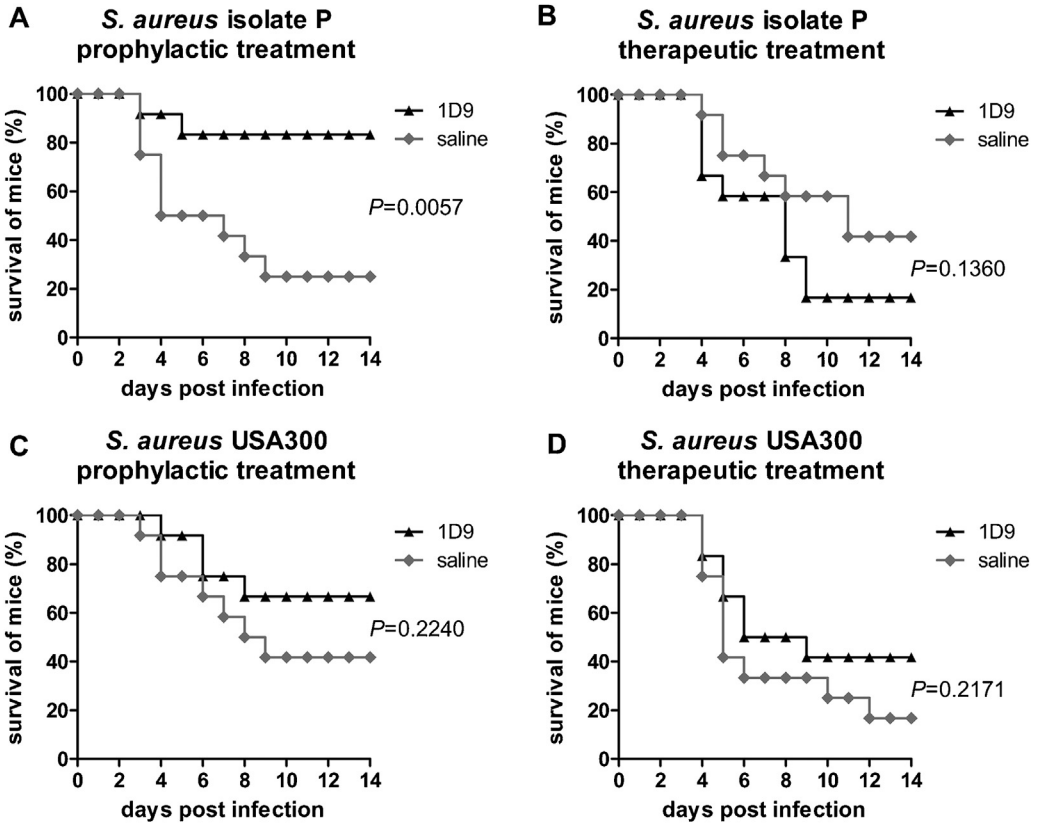

Fig. 7. Efficacy of 1D9 in mice with *S. aureus* bacteremia. Mice (12 per group) were infected with 2–4 × 10^5 CFU of *S. aureus* isolate P or with 4–7 × 10^5 CFU of *S. aureus* USA300, by intravenous inoculation. Animals were treated intravenously with 1D9 (5 mg/kg) or placebo (saline) 3 h before (A, C) or 3 h 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.

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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 (Lorenz et al., 2011) and hUK-66 (Oesterreich et al., 2014), 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 (Ziebandt et al., 2010), and *S. aureus* USA300 is one of the most frequent causes of community-acquired infections in the United States (McDougal et al., 2003). 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 h 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 (Dreisbach et al., 2010). 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 (Falugi et al., 2013). 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. (2011) 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 h later (Lorenz et al.) and 1 dose at 3 h 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. (2012) 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 (Benjamin et al., 2006; DeJonge et al., 2007; García-Lara and Foster, 2009; Ohlsen and Lorenz, 2010; Rupp et al., 2007; Weems et al., 2006). 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 (Kwakkenbos et al., 2010; Wrammert et al., 2008), 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. (2011) and Oesterreich et al. (2014) 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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Competing interests

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 Lanthio Pharma. 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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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijmm.2014.11.002.

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