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Protein A-neutralizing monoclonal antibody protects neonatal mice against *Staphylococcus aureus*

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Abstract

Staphylococcus aureus is a cause of sepsis and meningitis in very-low-birth-weight (VLBW) infants. Clinical trials with *S. aureus* specific antibodies failed to protect VLBW neonates, which may be due to the immune evasive attributes of staphylococcal protein A (SpA). Here we show that mouse monoclonal antibody SpA_{KKAA}-mAb 3F6, which neutralizes the immunoglobulin Fcγ-binding and B cell receptor crosslinking attributes of SpA, protects neonatal mice against *S. aureus* sepsis and raises protective immunity against subsequent staphylococcal infection. We developed a humanized SpA_{KKAA}-mAb that protects neonatal mice against *S. aureus* sepsis and may therefore be subjected to clinical testing in VLBW neonates.

Keywords

Staphylococcus aureus; Staphylococcal protein A; Monoclonal antibody; Neonatal bacteremia and meningitis; Protective immunity; Recurrent infection

1. Introduction

Approximately 60,000 infants per year in the United States are very-low-birth-weight (VLBW), defined as <1,500 g and requiring hospitalization and intensive care [1]. The risk of late-onset (> 72 hours following birth) bacteremia or meningitis with *Staphylococcus aureus* is increased in VLBW neonates with decreasing birth weight and gestational age. In a recent study, 3.6% of VLBW infants were diagnosed with *S. aureus* bacteremia and/or meningitis, which caused 26% mortality [2]. Neonatal sepsis is also associated with severe neurodevelopmental and growth defects [3]. Due to their low gestational age, VLBW

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Disclosure of potential conflicts of interest

Vilasack Thammavongsa, Hwan Keun Kim, Dominique Missiakas and Olaf Schneewind declare a conflict of interest as inventors of patent applications that are related to the development of *Staphylococcus aureus* vaccines and are currently under commercial license.

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neonates lack maternal immunoglobulin, which is thought to increase susceptibility to staphylococcal infection [4]. Clinical trials have examined the efficacy of human antibodies against staphylococcal capsule (Altastaph[®]), clumping factor A (ClfA) and serine-aspartate repeat protein G (SdrG) (INH-A21) or monoclonal antibody against polyglycerol-phosphate lipoteichoic acid (Pagibaximab[®]) to protect VLBW neonates against *S. aureus* infections [5–7]. Unfortunately, these trials failed to reach their study endpoints [8].

We hypothesize that the failure of antibodies to provide protection against *S. aureus* is based on the immune evasive attributes of staphylococcal protein A (SpA). Secreted SpA, which is either assembled in the envelope or released by bacteria, binds the Fc γ domain of immunoglobulins (Ig) as well as the Fab domains of V_H3-type IgG and IgM [9, 10]. The Fc γ binding activity of SpA enables *S. aureus* to escape opsonophagocytic killing, whereas the crosslinking of V_H3-type IgM B cell receptors disrupts the development of adaptive immune responses [11]. The non-toxicogenic variant SpA_{KKAA} is defective for immunoglobulin binding and, when used as immunogen, elicits SpA-neutralizing antibodies in mice and rabbits [12]. This enabled isolation of monoclonal antibodies, e.g. SpA_{KKAA}-mAb 3F6, which protect adult mice against *S. aureus* disease and provide adjuvant function for the development of antibodies against many different staphylococcal antigens [13]. Here we examined the efficacy of mouse and humanized SpA_{KKAA}-mAb to protect neonatal mice against *S. aureus* infection.

2. Materials and methods

2.1. Ethics statement

Experimental protocols were reviewed, approved and performed under supervision of The University of Chicago's Institutional Biosafety Committee (IBC) and Institutional Animal Care and Use Committee (IACUC). FVB albino mice, used for their large litter size, were obtained from Charles River Laboratories. Mice received antibiotic-free water and food ad libitum and dams delivered approximately 10 pups following a 21–22 day gestation period.

2.2. Bacterial Strains

S. aureus USA300 LAC, a methicillin-resistant clinical isolate (MRSA), was grown in tryptic soy broth (TSB) at 37°C. Overnight cultures of *S. aureus* were diluted 1:100 into fresh TSB and grown for 3 hours at 37°C. Staphylococci were centrifuged, washed twice and diluted in PBS to A₆₀₀ 0.5 (2×10^8 CFU ml⁻¹). Staphylococci were enumerated by colony formation on agar plates to quantify infectious doses.

2.3. Animal experiments

One-day-old pups were administered purified mAb SpA_{KKAA}-3F6 or control antibody via intraperitoneal injection. Twenty-four hours later, pups were infected by subcutaneous injection cephalad to their tail with 1×10^3 CFU *S. aureus* USA300 LAC in 200 μ l PBS. Pups were observed for survival and growth by weighing animals in daily intervals. Pups that survived the *S. aureus* challenge were weaned 21 days after birth; at 5 weeks of age, these mice were injected into the periorbital venous plexus with 5×10^6 CFU *S. aureus* USA300 LAC in 100 μ l PBS and monitored for survival.

2.4. Enzyme linked immunosorbent assay

ELISA plates were coated with affinity purified SpA_{KKAA} at 1 µg·ml⁻¹ in 0.1 M carbonate buffer (pH 9.5) at 4°C overnight. Plates were blocked and incubated with dilutions of hyperimmune sera and developed using OptEIA reagent (BD Biosciences). For inhibition of non-immune binding of human IgG to protein A, purified SpA, SpA_{KK} (Q⁹K and Q¹⁰K substitutions in each of the five IgBDs to abolish Fcγ binding) or SpA_{AA} (D³⁶A and D³⁷A substitutions in each of the five IgBDs to abolish Fab' binding) were used to coat ELISA plates [13]. Blocked plates were incubated with 50 µg·ml⁻¹ human IgG1 monoclonal antibody control or humanized SpA_{KKAA}-mAb prior to ligand binding. Plates were incubated with serial dilutions of human IgG conjugated to HRP and developed using OptEIA reagent. Half-maximal titers were calculated and normalized to human IgG1 control set at maximal binding.

2.5. Staphylococcal antigen matrix

Recombinant staphylococcal antigens were affinity purified - SpA_{KKAA}, clumping factor A (ClfA) and B (ClfB), iron-regulated surface determinant B (IsdB), Pantón-Valentin Leukocidin subunit F (LukF), coagulase (Coa) and von-Willebrand factor binding protein (vWbp) - and 2 µg each protein was blotted onto nitrocellulose membrane. Membranes were blocked with 5% whole milk, followed by incubation with hyper-immune sera (1:10,000 dilution). IRDye 680 conjugated affinity purified anti-mouse IgG (Rockland) was used to quantify signal intensities (A₇₀₀) using the OdysseyTM infrared imaging system (Li-cor).

2.6. Humanized SpA_{KKAA}-mAb

Humanized SpA_{KKAA}-mAb was derived from the mouse monoclonal antibody SpA_{KKAA}-mAb 3F6, which had been obtained by immunization of mice with SpA_{KKAA} [12]. Complementary determining regions (CDR) of SpA_{KKAA}-mAb 3F6 were grafted into a human IgG1 isotype antibody. The VH/VL sequence of SpA_{KKAA}-mAb was cloned into an expression vector containing the genetic elements optimized for enhanced expression of antibodies in the dihydrofolate reductase-deficient mutant Chinese hamster ovary (DHFR-CHO) cell line DG44. Humanized SpA_{KKAA}-mAb was affinity purified on protein A column and buffer exchanged into PBS.

3. Results

To develop a mouse model for *S. aureus* neonatal sepsis and efficacy testing of monoclonal antibodies, we bred FVB albino mice and obtained neonates that were infected via subcutaneous injection [14]. Forty-eight hours after birth, pups were assigned to specific experimental cohorts and injected with *S. aureus* USA300 LAC, the current epidemic MRSA strain in the United States. Initial experiments used infectious doses ranging from 1×10² to 1×10⁶ CFU. All pups that were infected with a dose greater than 10⁴ CFU ceased to gain weight and succumbed to *S. aureus* LAC challenge within 2–3 days (LD₅₀ dose 1×10³).

SpA_{KKAA}-mAb 3F6 is an IgG2a mouse monoclonal antibody that binds each of the five immunoglobulin-binding domains of SpA (E, D, A, B and C) and neutralizes their

association with mouse or human IgG and IgM [13]. SpA_{KKAA}-mAb 3F6 promotes opsonophagocytic killing of *S. aureus* LAC in mouse blood [13]; administration of 5 mg·kg⁻¹ of SpA_{KKAA}-mAb 3F6 to adult BALB/c mice prevents *S. aureus* USA300 LAC abscess formation in renal tissues [13]. To examine SpA_{KKAA}-mAb 3F6-mediated protection against neonatal sepsis in mice, 1-day-old pups received intraperitoneal injections with either SpA_{KKAA}-mAb 3F6 or IgG2a (5 mg·kg⁻¹ body weight) or were left untreated (mock)(Fig. 1A). Twenty-four hours later, on day-2 of life, pups were challenged by subcutaneous injection with 1×10³ CFU *S. aureus* USA300 LAC. Sixty-percent of mock-treated pups succumbed to *S. aureus* challenge over the five day observation period (Fig. 1B). Survival and time-to-death were not affected by intraperitoneal injection of IgG2a control antibody (Fig. 1B). Eighty percent of neonates that received SpA_{KKAA}-mAb 3F6 were protected against *S. aureus* USA300 LAC sepsis (mock vs. SpA_{KKAA}-mAb 3F6, P=0.0374; IgG2a vs. SpA_{KKAA}-mAb 3F6, P=0.0437). Pups that survived for 5 days did not show clinical signs of disease and developed similar to uninfected mice.

S. aureus infection in neonates, children or adults is not known to elicit protective immune responses and recurrent infection is a common feature of staphylococcal disease [11]. When examined in adult mice, *S. aureus* expression of SpA is required to block development of protective immune responses, which is attributable to its B cell superantigen activity [11]. We therefore examined whether neonatal mice that had survived *S. aureus* challenge were able to develop protective immune responses in the presence of SpA_{KKAA}-mAb 3F6. Serum IgG levels against seven *S. aureus* antigens previously shown to contribute to vaccine protection in mice-ClfA [15], ClfB [16], IsdB [17], SpA_{KKAA} [12] LukF [18], Coa and vWbp [19] were determined at 21 days post-challenge (Fig. 1C). As observed previously with adult BALB/c mice treated with SpA_{KKAA}-mAb 3F6 [13], neonatal FVB mice that had been immunized with SpA_{KKAA}-mAb 3F6 developed higher serum IgG titers against three *S. aureus* protective antigens: ClfA, IsdB and vWbp (Fig. 1C). In contrast to adult BALB/c mice immunized with SpA_{KKAA}-mAb 3F6, the IgG level against IsdB was significantly increased in serum samples of SpA_{KKAA}-mAb 3F6-immunized neonatal FVB mice (IgG2a vs. SpA_{KKAA}-mAb 3F6, P<0.0001, two-way ANOVA) [13]. Of note, high levels of anti-SpA_{KKAA} antibodies were detectable 21 days after passive transfer of SpA_{KKAA}-mAb 3F6, which may be derived from newly produced anti-SpA antibodies or circulating SpA_{KKAA}-mAb 3F6 (Fig. 1C). On day-35 of life, animals were challenged via intravenous injection with 5×10⁶ CFU *S. aureus* USA300 LAC, a dose that causes lethal sepsis in adult FVB mice. IgG2a isotype control-treated mice as well as naïve, age-matched mice all succumbed to the intravenous *S. aureus* challenge within 2–3 days (Fig. 1D). However, cohorts of mice that had received SpA_{KKAA}-mAb 3F6 on day-1 of life and had been infected 24 hours later displayed a significant delay in time-to-death and an increase in survival following challenge with an otherwise lethal dose of *S. aureus* USA300 LAC (IgG2a vs. SpA_{KKAA}-mAb 3F6, P=0.0072).

We humanized SpA_{KKAA}-mAb 3F6 and examined the half-life of circulating protein A specific antibody in neonatal mice. Following intraperitoneal injection of newborn mice with 10 mg·kg⁻¹ antibody, animals (n=4) were bled at variable time intervals (days 1, 3, 7 and 14) and serum concentration of SpA_{KKAA}-mAb 3F6 or humanized SpA_{KKAA}-mAb was

measured; this experiment revealed that humanized SpA_{KKAA}-mAb has a shorter half-life in mice than SpA_{KKAA}-mAb 3F6 (Fig. 2A). When examined for preclinical efficacy, intraperitoneal injection of humanized SpA_{KKAA}-mAb protected neonatal mice against staphylococcal sepsis, induced by subcutaneous injection of 1×10^3 CFU *S. aureus* USA300 LAC, whereas the human IgG1 control antibody did not (human IgG1 vs. humanized SpA_{KKAA}-mAb, $P=0.0439$) (Fig. 2B). Humanized SpA_{KKAA}-mAb bound with high affinity to SpA_{KKAA} (K_a $0.97 \text{ M} \times 10^{10} \text{ M}^{-1}$) and inhibited the association of protein A (SpA) with human IgG (Fig. 2C). The neutralizing activity of the humanized SpA_{KKAA}-mAb extends to both the Fc γ binding activity and the Fab V_{H3} crosslinking activity of protein A, as SpA_{KK} (only binds Fab V_{H3}) and SpA_{AA} (only binds Fc γ) association with human immunoglobulin G (hIgG) was inhibited by humanized SpA_{KKAA}-mAb but not by human IgG1 control (Fig. 2C).

4. Discussion

VLBW neonates are at increased risk for bacterial sepsis and meningitis, which is associated with high mortality and long-term disability [1]. *S. aureus* is a major cause of neonatal sepsis and meningitis that, in spite of significant efforts, can currently not be addressed with a specific preventive strategy [2]. Previous failure of antibodies to achieve protection could be caused by SpA, an immunoglobulin-binding factor of *S. aureus* that disables humoral immune responses of infected hosts [11]. To test this hypothesis, we developed a neonatal mouse model for efficacy testing of monoclonal antibodies against *S. aureus* sepsis, which is based on earlier work examining antibiotic prophylaxis in neonatal mice [14]. Here, subcutaneous injection of a 200 μl suspension of 1×10^3 CFU *S. aureus* USA300 LAC caused lethal sepsis, defined by replication of staphylococci in tissues of infected hosts and rapid mortality. Compared to mock or isotype IgG2a control treated animals, SpA_{KKAA}-mAb 3F6 protected neonatal mice against *S. aureus* sepsis. Moreover, the surviving mice that were treated with SpA_{KKAA}-mAb 3F6 as pups elicited protective immune responses against subsequent challenge with an otherwise lethal intravenous dose of *S. aureus* USA300 LAC. These findings are in agreement with SpA_{KKAA}-mAb 3F6 neutralization of SpA-dependent B cell superantigen activity during infection [13]. The SpA_{KKAA}-mAb 3F6 antibody was humanized by grafting complementarity determining regions onto human IgG1. Humanized SpA_{KKAA}-mAb 3F6 bound SpA_{KKAA} with high affinity, blocked IgG binding to protein A, and provided protection against *S. aureus* sepsis in neonatal mice.

The molecular and cellular mechanisms promoting SpA_{KKAA}-mAb 3F6 induced opsonophagocytosis of staphylococci in mice are not yet understood. For example, SpA_{KKAA}-mAb 3F6 induced protection may involve complement deposition, complement receptor-mediated uptake of *S. aureus* and eventual killing of the pathogen by either neutrophils or macrophages. Alternatively, SpA_{KKAA}-mAb 3F6 binding to protein A on the staphylococcal surface may promote Fc γ -receptor mediated uptake of the pathogen and killing by specific phagocyte populations [20]. The mouse model may provide important mechanistic insights into the establishment of protection against *S. aureus* that can subsequently be tested with human antibodies and human blood or phagocytic cells. To the best of our knowledge, data presented herein constitute the first demonstration that a monoclonal antibody can prevent *S. aureus* induced neonatal sepsis in animals and that it

can function as an adjuvant to elicit immune responses protective against subsequent infection. Humanized SpA_{KKAA}-mAb also protects neonatal mice against *S. aureus* sepsis. This antibody could therefore be tested in VLBW neonates to prevent *S. aureus* sepsis/ meningitis. Nevertheless, efficacy studies for SpA_{KKAA}-mAbs in the neonatal mouse model are currently limited to MRSA isolate LAC, a member of the non-capsulating USA300 clone epidemic in the United States [21]. Future work must examine SpA_{KKAA}-mAb efficacy against other MRSA isolates including capsule expressing strains. Further, SpA_{KKAA}-mAbs do not provide complete protection against *S. aureus* sepsis in mice, suggesting that additional antibodies may be needed to accomplish full protective immunity.

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Highlights

- Neonatal mouse model for vaccine testing to prevent *Staphylococcus aureus* sepsis
- Monoclonal antibody neutralizing protein A protects neonatal mice against sepsis
- Development of a humanized monoclonal antibody neutralizing protein A
- Humanized monoclonal antibody protects neonatal mice against sepsis

Fig. 1. Vaccination with SpA_{KKAA}-monoclonal antibody elicits protective immunity against *S. aureus* infection. (A) Experimental plan for the immunization of neonatal FVB albino mice with mouse SpA_{KKAA}-mAb 3F6 (intraperitoneal injection of 5 mg kg⁻¹), mouse IgG2a mAb (5 mg kg⁻¹ isotype control) or mock treatment, which was followed 24 hours later by subcutaneous MRSA challenge with *S. aureus* USA300 LAC (B); survivors were bled on day 21 and serum analyzed for *S. aureus* specific antibodies (C) and challenged on day 35 by intravenous injection with *S. aureus* USA300 LAC (D). (B) Kaplan-Meier survival analysis of neonatal FVB albino mice infected via subcutaneous injection of 1×10^3 CFU *S. aureus* USA300 LAC to examine the efficacy of antibody treatment in preventing lethal sepsis in neonatal mouse cohorts (n=16 for each treatment group). Data shown are representative of 3 independent experiments. Statistical significance was determined with the Mantel-Cox test: mock vs. SpA_{KKAA}-mAb 3F6, P=0.0374; IgG2a vs. SpA_{KKAA}-mAb 3F6, P=0.0437. (C) Serum samples of previously infected mice were analyzed for antibodies against ClfA (clumping factor A), ClfB (clumping factor B), IsdB (iron surface determinant B), SpA_{KKAA}, LukF (leukocidin F), Coa (coagulase), and vWbp (von Willebrand binding protein). IRDye 680 conjugated anti-mouse IgG was used to quantify signal intensities with the OdysseyTM infrared imaging system (Li-cor); brackets denote standard error of the means. (D) Kaplan-Meier survival analysis of 35 day old FVB albino mice that had been treated as described in (A&B) and received intravenous injection of 5×10^6 CFU *S. aureus*

USA300 LAC. Data shown are representative of 3 independent experiments. Statistical significance was determined with the Mantel-Cox test: mock vs. naïve, $P=0.1646$; mock vs. SpA_{KKAA}-mAb 3F6, $P=0.0054$; IgG2a vs. SpA_{KKAA}-mAb 3F6, $P=0.0072$.

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Fig. 2.

Humanized SpA_{KKAA}-mAb protects neonatal mice against *S. aureus* sepsis. (A) Following immunization via intraperitoneal injection with 10 mg kg⁻¹ humanized SpA_{KKAA}-mAb or 5 mg·kg⁻¹ mouse monoclonal antibody SpA_{KKAA}-mAb 3F6, neonatal FVB albino mice (n=4) were bled at timed intervals, serum concentration of antibodies determined by ELISA with SpA_{KKAA} antigen; brackets denote standard error of the means. (B) Neonatal FVB albino mice on day-of-life 1 were immunized by intraperitoneal injection with humanized SpA_{KKAA}-mAb (10 mg·kg⁻¹), human IgG1 control antibody (10 mg·kg⁻¹) or mock treated.

Neonates were challenged by subcutaneous injection of 1×10^3 CFU *S. aureus* USA300 LAC on day-of-life 2. Kaplan-Maier survival analysis to examine the efficacy of antibody treatment in preventing lethal sepsis in neonatal mouse cohorts (n=10 for each treatment group). Data shown are representative of 2 independent experiments. Statistical significance was examined with the Mantel-Cox test: mock vs. hSpA_{KKAA}-mAb, P=0.0114; hIgG1 vs. hSpA_{KKAA}-mAb, P=0.0439. (C) Purified SpA, SpA_{KK} or SpA_{AA} were coated onto ELISA plates and subjected to binding experiments with human IgG (hIgG) in the presence of either humanized SpA_{KKAA}-mAb (hSpA_{KKAA}-mAb) or human IgG1 control antibody (hIgG1). Brackets denoted standard error of the means.