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Preclinical Efficacy of Clumping Factor A in Prevention of Staphylococcus aureus Infection

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ABSTRACT Treatment of Staphylococcus aureus infections has become increasingly difficult because of the emergence of multidrug-resistant isolates. Development of a vaccine to prevent staphylococcal infections remains a priority. To determine whether clumping factor A (ClfA) is a good target protein for inclusion in a multivalent vaccine, we evaluated its efficacy in a variety of relevant staphylococcal infection models, challenging with different S. aureus strains. ClfA adsorbed to Alhydrogel and mixed with Sigma Adjuvant System was more immunogenic and stimulated a more robust Th17 response than ClfA administered with alum alone. ClfA immunization induced the production of functional antibodies in rabbits and mice that blocked S. aureus binding to fibrinogen and were opsonic for S. aureus strains that produced little or no capsular polysaccharide. Mice immunized with ClfA showed a modest reduction in the bacterial burden recovered from subcutaneous abscesses provoked by S. aureus USA300 strain LAC. In addition, the ClfA vaccine reduced lethality in a sepsis model following challenge with strain Newman, but not ST80. Vaccination with ClfA did not protect against surgical wound infection, renal abscess formation, or bacteremia. Passive immunization with antibodies to ClfA did not protect against staphylococcal bacteremia in mice or catheter-induced endocarditis in rats. Some enhancement of bacteremia was observed by ClfA immunization or passive administration of ClfA antibodies when mice were challenged by the intraperitoneal route. Although rodent models of staphylococcal infection have their limitations, our data do not support the inclusion of ClfA in an S. aureus multivalent vaccine.

IMPORTANCE Antibiotics are often ineffective in eradicating Staphylococcus aureus infections, and thus, a preventative vaccine is sorely needed. Two single-component vaccines and two immunoglobulin preparations failed to meet their designated endpoints in phase III clinical trials. Importantly, recipients of an S. aureus surface protein (iron surface determinant B) vaccine who developed a staphylococcal infection experienced a higher rate of multiorgan failure and mortality than placebo controls, raising safety concerns. Multicomponent S. aureus vaccines have now been generated, and several include surface protein clumping factor A (ClfA). We immunized mice with ClfA and generated a robust T cell response and serum antibodies that were functional in vitro. Nonetheless, ClfA was not protective in a number of relevant animal models of S. aureus infection, and high levels of ClfA antibodies enhanced bacteremia when mice were challenged with community-acquired methicillin-resistant S. aureus strains. Evidence supporting ClfA as a vaccine component is lacking.

S. aureus is a Gram-positive, extracellular bacterium that causes both superficial and invasive infections, such as abscesses, sepsis, bacteremia, and endocarditis (1). It is among the most frequently isolated bacterial pathogens in hospitals, and during the past decade, community-acquired methicillin-resistant S. aureus (CA-MRSA) strains with high virulence have infected individuals without underlying risk factors (1, 2). Treatment of staphylococcal infections has become increasingly difficult because of the emergence of multidrug-resistant strains (3, 4). As such, development of a vaccine to prevent S. aureus infections remains a priority.

S. aureus expresses a broad range of cell surface proteins that play important roles during the pathogenesis of S. aureus-caused disease (5, 6). Many of these proteins are anchored to the cell wall of S. aureus by the enzyme sortase A (7); they modulate bacterial adherence to host cells by engaging host extracellular matrix molecules, such as fibronectin, collagen, and fibrinogen (Fg). Cell wall-anchored protein A binds to the Fc fragment of IgG and to the Fab portion of V\(_{H}\)-3-type B cell receptors (8), resulting in bacterial evasion of the host immune response (6, 8).

S. aureus clumping factor A (ClfA) is a major staphylococcal adhesin. ClfA binds to dimeric host Fg through the carboxy-terminal domain of the Fg gamma chain, resulting in bacterial aggregation in plasma or in purified Fg (9). As the major Fg bind-
ing protein, ClfA mediates staphylococcal binding to immobilized Fg- or fibrin-coated surfaces, promoting bacterial adherence to blood clots and biomaterials (9, 10). ClfA also binds to complement factor I, resulting in cleavage of the complement opsonin C3b (11, 12). In addition, ClfA has been reported to bind in vitro to serum apolipoprotein E (13) and human platelets in a fibrinogen-independent manner (14).

The full-length ClfA protein comprises an N-terminal Fg binding domain (the A region), followed by a variable number of serine-aspartate dipeptide repeats, a sorting signal, and a C-terminal wall-spanning region (6, 9). The N-terminal A region is composed of three separately folded domains: N1, N2, and N3. The N2 and N3 subdomains of ClfA (ClfAN23; amino acids [aa] 221 to 559) form IgG-like folds that bind Fg (6, 15), whereas the N1 subdomain (aa 40 to 220) is required for export and cell wall localization (16). Recently, the serine-aspartate repeats were shown to be modified by O-linked N-acetylgalactosamine residues (17, 18).

ClfA is recognized as an important S. aureus virulence factor, since it has been shown to enhance staphylococcal virulence in experimental models of septic arthritis (19), sepsis (20), endocarditis (10), and skin infection (21). Josefsson et al. (22) constructed a mutant ClfA protein that no longer binds to Fg by mutating P336 and Y338 to serine and alanine, respectively. An S. aureus strain producing the ClfA mutant protein was attenuated in murine models of septic arthritis, lethality, and abscess formation (21, 22).

Moreover, Flick et al. (23) showed that mice that produced Fg lacking the ClfA binding motif showed a consistent survival advantage compared to wild-type mice when challenged intravenously (i.v.) with a lethal dose of S. aureus Newman or USA300 FPR3757.

Preclinical studies testing ClfA as a vaccine antigen showed modest protection in rodent models of S. aureus septic arthritis, lethality, endocarditis, and abscess formation, but not osteomyelitis (19, 22, 24–26). Inhibitex first targetted ClfA in an immunotherapeutic approach to prevent S. aureus infection in humans. INH-21 (Veronate) was a pooled human immunoglobulin preparation from donors with high antibody titers against two Fg binding proteins—ClfA from S. aureus and SdrG from Staphylococcus epidermidis. In a phase III clinical trial, neonates received either placebo or 750 mg of INH-A21/kg of body weight, but the product failed to protect against late-onset sepsis by S. aureus or S. epidermidis (27, 28). Tefibazumab (Aurexis), a humanized monoclonal antibody that binds ClfA, was evaluated in a phase II trial in hospitalized patients with documented S. aureus bacteremia who received either a single tefibazumab dose plus standard therapy or standard therapy alone (29). To evaluate efficacy, a composite clinical endpoint was used, consisting of a relapse of S. aureus bacteremia, a complication related to bacteremia, or death. In the tefibazumab group, 2 of 30 patients reached the clinical endpoint compared with 4 of 30 patients in the placebo group ($P = 0.455$). The most notable vaccine failure was the cell wall-anchored S. aureus protein antigen iron surface determinant B, which was compared to a placebo in patients prior to elective cardiothoracic surgery with median sternotomy. The IsdB vaccine did not reduce the rate of serious postoperative staphylococcal infections, but it was associated with a significantly higher rate of multiorgan failure and mortality among vaccine recipients who developed S. aureus infections (30).

Subsequent to these clinical failures and those of other vaccine antigens (30, 31), the focus toward vaccine development against S. aureus has moved toward development of a multicomponent vaccine, particularly one that elicits not only a Th2 response but also Th1 and Th17 responses in the host. ClfA was included in vaccines that reached early clinical trials sponsored by both Pfizer (32) (ClinicalTrials.gov NCT01018641) and GlaxoSmithKline (33) (ClinicalTrials.gov NCT01160172), and ClfA is included in the ongoing Pfizer phase Ib trial of SAA4Ag vaccine in humans having elective lumbar spinal fusion procedures (NCT02388165). However, neither group has published preclinical data showing that ClfA was protective in relevant models of S. aureus infection. Recently, scientists from Pfizer reported that immunization with recombinant ClfA protected mice from lethality induced by 10$^6$ CFU of a Lactococcus lactis strain that was engineered to produce abundant ClfA on its surface (34). Their report did not include challenge with S. aureus, a pathogenic microbe with many virulence determinants that allow it to adhere to and damage host tissues and evade the immune system.

To further evaluate the impact of immunization with S. aureus ClfA on staphylococcal disease, we investigated its efficacy as a protective immunogen in a variety of relevant staphylococcal infection models. In our investigations, we challenged the animals with several different S. aureus strains. Our results do not confirm those of other groups who reported that ClfA was protective in preventing staphylococcal disease.

RESULTS

ClfA immunogenicity. Mice were actively immunized with the N1, N2, and N3 subdomains of ClfA (ClfAN123) (aa 40 to 999) or ClfAN23 (aa 221 to 559) at doses ranging from 2 μg to 30 μg per mouse. Control animals were vaccinated with either bovine serum albumin (BSA) or an irrelevant Shigella flexneri 2a O antigen conjugated to Pseudomonas aeruginosa exoprotein A (2a-Epa) vaccine. When the ClfA protein (2, 20, or 30 μg) was adsorbed to alum, the antibody responses elicited by ClfAN123 and ClfAN23 were fairly comparable (Fig. 1). The antibody levels were enhanced when the ClfA protein (adsorbed to alum) was mixed with Sigma Adjuvant System (SAS) prior to immunization. As shown in Fig. 1, the optimal dose for either ClfAN123 or ClfAN23 was 2 μg/mouse.

![ClfA-specific antibody levels in sera from mice immunized on days 0, 14, and 28 with various quantities of ClfAN123, ClfAN23, or BSA, as determined by ELISA at a 1:100 serum dilution. Solid lines depict mice immunized with vaccines adsorbed to alum and then mixed with SAS. Dashed lines depict mice immunized with vaccines adsorbed to alum only.](image-url)

**Fig 1.** ClfA-specific antibody levels in sera from mice immunized on days 0, 14, and 28 with various quantities of ClfAN123, ClfAN23, or BSA, as determined by ELISA at a 1:100 serum dilution. Solid lines depict mice immunized with vaccines adsorbed to alum and then mixed with SAS. Dashed lines depict mice immunized with vaccines adsorbed to alum only.
T cell-derived interleukin 17 (IL-17) and interferon gamma (IFN-γ) play important roles in host clearance of experimental infection of mice with *S. aureus* (35, 36). When we compared the T cell response of mice vaccinated with ClfA adsorbed to alum with that of mice vaccinated with ClfA adsorbed to alum and then mixed with SAS (ClfA plus alum/SAS) prior to subcutaneous (s.c.) injection, only the latter group elicited a demonstrable T cell response in the immunized mice. Lymph node cells recovered from mice given ClfA adsorbed to alum did not produce IFN-γ or IL-17 in response to *in vitro* stimulation with ClfA (Fig. 2A and B), nor could we detect significant intracellular cytokine staining compared to controls (Fig. 2C and D). However, cells from mice immunized with ClfA plus alum/SAS secreted significantly more IFN-γ and IL-17 than did cells from control mice injected with Tris-buffered saline (TBS) plus alum/SAS (Fig. 2E and F). IL-4 secretion could not be detected in any group of mice (not shown). Intracellular cytokine staining experiments were performed with *in vitro*-stimulated cells to determine whether the cytokines were derived from CD4+ T cells. As shown in Fig. 2G and H, vaccination of mice with ClfAN23 plus alum/SAS resulted in a significant increase in the numbers of both Th1 (CD4+ IFN-γ-positive [IFN-γ⁺]) and Th17 (CD4+ IL-17-positive [IL-17⁺]) cells in the draining lymph nodes compared to the control mice, indicating that immunization of mice with ClfA adsorbed to alum plus SAS elicited both Th1 and Th17 immune responses.

**Inhibition of *S. aureus* binding to Fg by anti-ClfA IgG.** *S. aureus* binds to Fg, and ClfA is the major Fg binding protein (9). To determine whether antibodies elicited by ClfA vaccination were functional, we assessed whether the ClfA antibodies would block *S. aureus* binding to Fg immobilized on a 96-well plate. As shown
in Fig. 3, the binding of *S. aureus* Newman Δspa mutant strain to human Fg was blocked in a dose-dependent fashion by serial dilutions of either rabbit IgG to ClfA (Fig. 3A) or serum from mice immunized with ClfA (Fig. 3B). Similar results were obtained in blocking experiments performed with the wild-type Newman strain (not shown). In contrast, control antibodies to capsular polysaccharide type 8 (CP8)-Epa (Fig. 3A) or *S. aureus* HLa (Fig. 3B) showed no Fg binding inhibition. These results indicate that antibodies to ClfA can functionally block the binding of *S. aureus* surface-associated ClfA to human Fg.

**Opsonic activity of antibodies to ClfA.** Antibodies to *S. aureus* capsular polysaccharides serotypes 5 and 8 have been shown to mediate opsonophagocytic killing (OPK) of encapsulated *S. aureus* strains (37–40). Moreover, Nanra et al. reported that antibodies to ClfA were opsonic against encapsulated *S. aureus* (41). To determine whether the ClfA antibodies utilized in our study were opsonic, we performed OPK assays with HL60 phagocytic cells, serum complement, and antibodies to CIAN123 or CIAN23. As shown in Fig. 4A, rabbit IgG raised to either CIAN23 or CIAN123 was opsonic for the acapsular CA-MRSA strain LAC (USA300). IgG concentrations of 10 μg/ml resulted in ≥50% killing, and the opsonic activities of CIAN23 and CIAN123 IgG were not significantly different at the lower antibody concentrations. In contrast, rabbit IgG to CIAN23 was not opsonic for strains that were encapsulated, e.g., the serotype 8 strain Reynolds (CP8) and ST80 and the serotype 5 strain Newman (Fig. 4B). Control CP8-Epa antibodies were opsonic for the CP8+ strains Reynolds (CP8) and ST80 (Fig. 4B) but not for the CP5+ strain Newman (not shown). As expected, the irrelevant control IgG to Shigella 2a-Epa was not opsonic.

We then tested sera from mice actively immunized with CIAN23 plus alum/SAS for opsonic activity. As shown in Fig. 4C, strain LAC was susceptible to OPK (>50% killing of the input inoculum) at mouse serum dilutions as high as 1:3,240 (Fig. 4C). The opsonic titers of the mouse CIAN23 antiserum were substantially lower against the encapsulated isolates—1:360 against the serotype 8 strain ST80 and 1:40 against the serotype 5 strain Newman. The ClfA antiserum showed no opsonic activity against the heavily encapsulated Reynolds (CP8) strain. Serum antibodies to the CP8-Epa conjugate vaccine were opsonic for both CP8+ strains ST80 and Reynolds (CP8), whereas antiserum to the heterologous control vaccine (2a-Epa) was poorly opsonic for all four strains tested. Of note, the acapsular strain LAC showed some nonspecific background killing activity (5 to 25% reduction in the number of CFU/milliliter) with all of the IgG and serum samples tested.

**Efficacy of ClfA immunization for the prevention of *S. aureus* infections.** *S. aureus* is a major cause of skin and soft tissue infections, and Kwiecinski et al. showed that a ClfA mutant strain of *S. aureus* that lacked Fg binding showed attenuated virulence in an s.c. abscess infection model (21). These results prompted us to evaluate the ability of immunization with a ClfA vaccine (mutated to abrogate Fg binding) to prevent s.c. abscess formation.

We actively immunized groups of mice with the optimally immunogenic dose of 2 μg CIAN123, CIAN23, or BSA, and each vaccine preparation was administered with alum and SAS. Two weeks after the last immunization, *S. aureus* LAC was mixed with Cytodex beads, and the suspension was injected by the s.c. route into the mouse flanks. Abscesses provoked by strain LAC are not necrotic in this infection model, and we aseptically excised...
them for quantitative culture 2 days after bacterial challenge. As shown in Fig. 5A, mice immunized with ClfAN23 showed a modest but significant reduction in the abscess bacterial burden compared to the control group immunized with BSA. Immunization with ClfAN123 had no effect, and there were no differences between the groups for abscess weight (Fig. 5B) or mouse weight loss (Fig. 5C).

Timofeyeva et al. (42) demonstrated that ClfA is produced in vivo in an S. aureus surgical wound infection model, although they observed considerable strain-to-strain variation. In initial experiments, we immunized mice with 30 μg ClfAN23 or ClfAN123 absorbed to alum; on day 42, the animals were subjected to surgical wound infection. Three days after challenge with 90 CFU of S. aureus Newman, the tissue bacterial burden and mouse weight loss were evaluated. Immunization with ClfA provided no protection against staphylococcal wound infection (Fig. 6A) or associated weight loss (Fig. 6B). Additional mice were vaccinated with 2 μg ClfAN23 (mixed with alum and SAS) because this dose resulted in a robust antibody response and elicited a Th1 and Th17 response in the animals (Fig. 2). The immunized mice were challenged with 150 to 300 CFU of S. aureus Newman. As shown in Fig. 6C and D, we observed no additional protection against surgical wound infection or weight loss in mice vaccinated with ClfA plus alum/SAS.

S. aureus is a major cause of sepsis, a systemic inflammation that arises from bacteremia and affects multiple organs; sepsis often results in a lethal outcome (43). Previous studies indicated that BALB/c mice immunized with 10 μg ClfAN123 administered with alum (24) or 50 μg ClfAN123 mixed with Freund’s adjuvant (20) reduced lethality following challenge with S. aureus 834 or Newman, respectively. Accordingly, we evaluated ClfA as a vaccine candidate in the lethality model. Mice immunized with ClfAN23, 2a-Epa, or BSA were challenged intravenously (i.v.) with ~10⁸ CFU of S. aureus strain Newman or ST80, a serotype 8 CA-MRSA isolate prevalent in Europe (44). Strain Newman appeared more virulent than ST80 in this model, and ClfAN23 immunization increased the survival time in mice challenged with strain Newman (Fig. 7A). However, vaccination with ClfA did not enhance survival in mice challenged with strain ST80 (Fig. 7B), indicating the strain specificity of protection in this model.

Narita et al. also reported that immunization with ClfAN123 and alum significantly reduced the bacterial burden in the kidneys and spleens of mice challenged i.v. with a sublethal dose of the Japanese isolate 834 (24). We immunized mice with ClfAN23 or BSA and challenged them with either S. aureus ST80 or LAC. On day 3, we evaluated the tissue bacterial burdens and weight loss. ClfA vaccination failed to reduce the bacterial burden in the mouse kidneys (Fig. 8A and D) or spleen (Fig. 8B and E). Weight loss for both groups was similar (Fig. 8C and F).

Because ClfA is produced in vivo in the murine bacteremia infection model (42), we assessed whether immunization with ClfA might reduce bacteremia. In the initial studies, mice were

![FIG 5](image-url) Protective efficacy of ClfA in the mouse model of s.c. abscess formation. Mice were actively immunized with 2 μg ClfAN123, ClfAN23, or BSA adsorbed to alum and mixed with SAS. Two weeks after the third immunization, the animals were challenged s.c. with 4 × 10⁶ CFU of S. aureus LAC. (A to C) Number of CFU/abscess (A), abscess weight (B), and mouse weight change (C) were determined 2 days after challenge. The Kruskal-Wallis test showed a significant (P = 0.0306) difference among the three groups in panel A. The values for the BSA and ClfAN23 groups were significantly different (P = 0.0369) by Dunn’s multiple-comparison posttest and are indicated by a bar and asterisk. Each symbol represents the value for an individual animal, and the median value for each group is indicated by a horizontal line. The lower limit by quantitative culture is indicated by a dotted line. Data are representative of two independent experiments.

![FIG 6](image-url) Protective efficacy of ClfA immunization in the surgical wound infection model. Mice immunized with 30 μg ClfAN23, ClfAN123, or 2a-Epa (adsorbed with alum) were inoculated with 90 CFU of S. aureus Newman. (A and B) Bacterial burden (A) and weight change (B) were evaluated 3 days after bacterial challenge. Data are representative of three independent experiments. In a separate study, mice actively immunized with 2 μg ClfAN23 or BSA (mixed with alum and SAS) were challenged with 150 to 300 CFU of strain Newman. (C and D) The tissue bacterial burden (C) and weight change (D) were measured on day 3. k. Each symbol represents the value for an individual animal, and the median value for each group is indicated by a horizontal line. The lower limit by quantitative culture is indicated by a dotted line. The data are pooled from two independent experiments.
immunized with either 2 μg ClfA plus alum or BSA plus alum and challenged intraperitoneally (i.p.) with strain Reynolds (CP8) 2 weeks after the third immunization. The results of quantitative blood cultures performed 2 h after bacterial challenge were not significantly different between the two groups (P = 0.1044), although the mice given ClfA showed an overall increase in the number of CFU/milliliter of blood (see Fig. S1A in the supplemental material). None of the mice were still bacteremic on day 5, and kidney cultures were significantly higher (P < 0.05) in the mice given ClfA (Fig. S1B). Changes in mouse weight on day 5 were similar between animals vaccinated with ClfA or BSA (Fig. S1C).

To enhance the possible efficacy of ClfA as a protective immunogen in the bacteremia model, we immunized additional groups of mice with 2 μg BSA or ClfAN23 (with alum plus SAS) and challenged them with S. aureus strains that produce little or no capsular polysaccharide. Quantitative blood culture performed 1.5 h following bacterial challenge with the acapsular strain LAC revealed that mice vaccinated with ClfAN23 showed significantly higher bacteremia levels than control mice given BSA (Fig. 9A). To determine whether the adverse outcome of ClfA
immunization was unique to strain LAC, additional mice were immunized and challenged with ST80, which produces trace amounts of CP8 (B. Liu and J. C. Lee, submitted for publication). Mice vaccinated with ClfAN23 showed bacteremia levels similar to those of mice given BSA (Fig. 9B). Likewise, significant differences were not observed in the kidneys, hearts, or spleens of mice immunized with ClfA or BSA and challenged with strain ST80 (data not shown).

**Efficacy of passive immunization with antibodies to ClfA for the prevention of S. aureus bacteremia.** Because of the elevated LAC bacteremia levels that we observed following active immunization with ClfA, we performed additional bacteremia studies in which naive mice were passively immunized with rabbit IgG. Each mouse received 1 mg of either ClfAN23 IgG or Shigella 2a-Epa IgG by the i.v. route 24 h prior to bacterial challenge by the i.p. route. As shown in Fig. 10, the bacteremia results were dependent upon the S. aureus challenge strain. Mice that received ClfAN23 IgG showed significantly higher bacteremia levels when challenged with either strain LAC (Fig. 10A) (P < 0.0001) or ST80 (Fig. 10B) (P < 0.05). In contrast, passive immunization had no protective or deleterious effect when the mice were challenged with the serotype 5 Newman strain (Fig. 10C). Moreover, the Newman ΔclfA mutant was not attenuated in the bacteremia model, since mice challenged with the mutant strain showed bacteremia levels similar to those of the parental strain Newman.

We considered the possibility that antibodies to ClfA might be preventing Fg-mediated clumping of S. aureus LAC within the peritoneal cavity, allowing S. aureus to reach the bloodstream in higher numbers following i.p. challenge. Therefore, we assessed the bacterial load in both mouse blood and peritoneal washes 1.5 h after S. aureus LAC challenge by the i.p. route. Although mice given antibodies to ClfAN23 had significantly (P = 0.026) higher levels of S. aureus recovered from blood samples (median of 4.51 log10 CFU/ml) than mice given 2a-Epa antibodies (2.49 log10 CFU/ml), similar numbers of staphylococci were recovered from the peritoneal wash fluid samples (medians of 6.18 versus 5.81 log10 CFU/ml for the anti-ClfA and anti-2a-Epa groups, respectively). Plating the peritoneal wash fluid samples following sonication increased the number of CFU/milliliter recovered by a factor of 4- to 5-fold for each mouse group (not shown).

Additional groups of mice were passively immunized before i.v. challenge with 1.2 × 108 CFU S. aureus LAC. Under these conditions, there was no difference in the 4 h postinfection bacteremia levels between mouse groups given antibodies to ClfA or 2a-Epa (Fig. 10D). The mice were euthanized on day 3. At that time point, the bacterial levels in blood samples (Fig. 10E), spleens (Fig. 10F), and kidneys (Fig. 10G) of the mice challenged i.v. were similar for mice given ClfA or 2a-Epa IgG.

**Efficacy of passive immunization with antibodies to ClfA for the prevention of S. aureus endocarditis.** Catheterization of rats results in deposition of platelet-fibrin thrombi on the aortic valves, resulting in sterile vegetations (45). When S. aureus is introduced into the bloodstream, it adheres to the damaged valves via the interaction between surface-associated ClfA, platelets, and fibrin (10). Moreillon et al. (10) reported that a clfA mutant of strain Newman was less virulent than the parental strain in the rat model of catheter-induced endocarditis. Moreover, Patti (46) showed that catheterized rabbits treated with ClfA antibodies and vancomycin showed improved bacterial clearance from blood compared to rabbits treated with vancomycin treatment alone.

Accordingly, we passively immunized catheterized rats with 3 mg IgG against ClfAN123, ClfAN23, or 2a-Epa prior to bacterial challenge with S. aureus Newman. Bacteremia was measured daily by quantitative cultures of blood samples, and surviving rats were sacrificed 72 h postinfection. Bacteremia, weight loss, vegetation size, and the bacterial burden in the vegetations and kidneys were measured.

FIG 10 Protective efficacy of passive immunization with antibodies to ClfA in the mouse bacteremia model. Mice passively immunized i.v. with 1 mg IgG purified by protein A affinity chromatography from rabbits immunized with ClfAN23 or 2a-Epa. (A to C) The following day, the animals were challenged i.p. with S. aureus LAC (A), ST80 (B), or Newman (C). Data presented in panels A and B are pooled from three and two independent experiments, respectively. In panel C, mice were challenged with the wild-type (wt) strain Newman or its isogenic ΔclfA mutant (DU5852). (D to G) Mice passively immunized with 1 mg IgG to ClfA or 2a-Epa were challenged i.v. with S. aureus LAC. Bacteremia was measured 4 h (D) and 3 days (E) after bacterial challenge, and the bacterial burden in the spleen (F) and kidneys (G) was measured 3 days after challenge. Values for the treatment group that are statistically significantly different from the value for the control group administered 2a-Epa IgG are indicated as follows: **, P < 0.01; ****, P < 0.0001.
assessed. Neither antibodies to ClfAN123 or ClfAN23 protected rats against catheter-induced endocarditis provoked by strain Newman. Compared to the control group of rats that received the irrelevant *Shigella* 2a-Epa IgG, the rats that received either ClfAN123 or ClfAN23 IgG showed similar results for each parameter of the infection. There were no significant differences between the groups in the vegetation weights (Fig. 11A), and the bacterial burdens in the vegetations (Fig. 11B) and kidneys (Fig. 11C) were comparable. Rats given ClfAN23 IgG showed modestly enhanced survival, but the difference between the groups was not significant (Fig. 11D). Bacteremia levels (Fig. 11E) and weight change (Fig. 11F) over time were similar among the three rat groups.

**DISCUSSION**

*S. aureus* ClfA has been regarded as a prime vaccine candidate because it is produced by most clinical isolates of *S. aureus*, it is surface associated, and it mediates staphylococcal binding to host Fg and fibrin. Moreover, clfA mutants of *S. aureus* have been shown to be less virulent in preclinical models of lethality, renal abscess formation, and septic arthritis (21, 22), attesting to the importance of ClfA in the pathogenesis of staphylococcal infection.

The majority of ClfA efficacy studies reported in the literature utilized Freund’s adjuvant in the immunization regimen, an adjuvant too reactogenic for use in humans (47). Josefsson et al. reported that mice immunized with 30 μg ClfA and Freund’s adjuvant showed reductions in arthritis and lethality in a septic arthritis model, and septic arthritis (21, 22), attesting to the importance of ClfA in the pathogenesis of staphylococcal infection.

FIG 11 Passive immunization with rabbit IgG specific for ClfAN23 or ClfAN123 failed to protect rats against infective endocarditis induced by *S. aureus* Newman. (A to D) Vegetation weight (A), number of CFU/vegetation (B), kidney bacterial burden (C), and survival (D) were assessed on day 3. (E and F) Bacteremia (E) and weight change (F) were analyzed at 24, 48, and 72 h after bacterial challenge. Horizontal lines represent group medians. The lower limit of detection by culture is indicated by a dotted line. The depicted data are pooled from three independent experiments.

The majority of ClfA efficacy studies reported in the literature utilized Freund’s adjuvant in the immunization regimen, an adjuvant too reactogenic for use in humans (47). Josefsson et al. reported that mice immunized with 30 μg ClfA and Freund’s adjuvant showed reductions in arthritis and lethality in a septic arthritis model, but protection was dependent on the strain, and the tissue bacterial burdens were not reduced by immunization (19). Likewise, mice immunized with 30 μg of the ClfAPY mutant protein (that lacks Fg binding) mixed with Freund’s adjuvant were protected against lethality induced by strain Newman, but reductions in arthritis were not significant (22). Therapy with ClfA antibodies and vancomycin resulted in better bacterial clearance from the blood of rabbits with catheter-induced *S. aureus* endocarditis than vancomycin treatment alone (48). However, the bacterial burdens in the tissues of the infected animals were not significantly reduced in these studies. Stranger-Jones et al. (25) immunized mice by the intramuscular route with 100 μg ClfA mixed with Freund’s adjuvant. Compared to mice given phosphate-buffered saline (PBS), ClfA immunization significantly reduced the bacterial burden in the kidneys 4 days after retroorbital challenge with *S. aureus* Newman (25). Similarly, McAdow et al. showed that immunization with 50 μg ClfA and Freund’s adjuvant increased the survival time of mice challenged with a lethal dose of strain Newman (20). However, the same investigators reported that ClfA antibodies did not protect against lethality induced by *S. aureus* strain N315 or MW2 (20).

Other vaccine studies have administered ClfA with alum, an adjuvant approved for use in humans. Narita et al. immunized BALB/c mice with 10 μg ClfAN123 adsorbed to alum and demonstrated protection against a lethal challenge with *S. aureus* 834, a clinical sepsis isolate from Japan (24). The same study reported a reduction in the bacterial burden in the kidneys and spleens of mice immunized with ClfA adsorbed to alum and challenged i.v. with a sublethal dose of strain 834 (24). In a GlaxoSmithKline-supported study, immunization with 20 μg ClfAN123 adsorbed to alum was not efficacious in protecting rats from experimental osteomyelitis when the animals were challenged with two different strains of *S. aureus* (26). In the most recent study, Scully et al. immunized mice with an undisclosed quantity of ClfAN123 ad-
sorbed to alum and demonstrated that this protected the animals for 24 h from lethality induced by i.v. challenge with 10^6 CFU of an L. lactis strain expressing plasmid-encoded ClfA (34). Passive immunization with 1.5 mg of ClfA-specific monoclonal antibody 12-9 also protected mice against lethal L. lactis-ClfA challenge in mice. However, the Scully et al. studies do not support the efficacy of ClfA as a vaccine candidate against the pathogen S. aureus.

Our study was designed to evaluate ClfA as a protective immunogen in the absence of Freund’s adjuvant and in mouse models of S. aureus infection that are relevant to human disease. Initially, we compared the immunogenicity of ClfAN123 and ClfAN23. Because ClfAN123 is readily degraded to ClfAN23 when expressed in E. coli, ClfAN23 was utilized for the majority of our experiments. Only ClfAN23 has been crystallized for structural analysis (49), and its immunogenicity in mice was similar to that of ClfAN123 in our hands. We report that immunization with ClfA mixed with alum did not induce a Th1 or Th17 response in C57BL6/J mice, but the addition of a monophosphoryl lipid A (MPL)-containing adjuvant (SAS) did induce both a high antibody response and CD4+ lymph node cells that produced IFN-γ and IL-17 upon in vitro stimulation with ClfA. The antibodies elicited by immunization with ClfAN23 showed in vitro functionality because they prevented the binding of S. aureus to immobilized Fg and were opsonic for acapsular S. aureus in an OPK assay.

We immunized mice under what we determined to be optimal conditions (2 μg ClfAN23 adsorbed to alum plus SAS) before evaluating protective efficacy in murine models of S. aureus disease. Our positive results include a modest but significant (0.5-log-unit) reduction in bacterial numbers recovered from subcutaneous abscesses of mice immunized with ClfA. However, protection was observed only in mice given ClfAN23, not in mice vaccinated with ClfAN123. In addition, we were able to achieve a significant increase in survival time in mice immunized with ClfAN23 and challenged with a lethal dose of strain Newman, confirming the results reported by other groups (12, 20, 22). However, ClfA-mediated protection against lethality was not achieved in immunized mice challenged with S. aureus strain ST80, confirming the strain specificity observed previously by another group (20).

Vaccination with ClfA or ClfA IgG was not protective in rodent models of surgical wound infection, cather-induced endocarditis, or the sublethal i.v. challenge model that results in renal abscess formation. Of note was the enhanced bacteremia that we observed following i.p. challenge with strain Newman, confirming the results reported by other groups (12, 20, 22). However, ClfA-mediated protection against lethality was not achieved in immunized mice challenged with S. aureus strain ST80, confirming the strain specificity observed previously by another group (20).

MATERIALS AND METHODS

Clumping factor A production and purification. Clumping factor A (ClfA) variants and the control Shigella flexneri 2a O antigen conjugated to Pseudomonas aeruginosa exoprotein A (2a-Epa) were produced and provided by GlycoVaxyn AG, Schlieren, Switzerland. Open reading frames (ORFs) encoding ClfA P336SY338A (mutations that abrogate ClfA binding to fibrinogen [Fg] [22]) comprising strain Newman N-terminal domains 1, 2, and 3 (ClfAN123) (aa 40 to 539) or N-terminal domains 2 and 3 (ClfAN23) (aa 221 to 539) and carrying a C-terminal six-His affinity tag were cloned into a pEC415 expression vector (53) after an OmpA signal sequence. ClfA variants were expressed in Escherichia coli W3110 in shacking flasks with super optimal or terrific broth medium with 100 µg/ml sorbed to alum and demonstrated that this protected the animals for 24 h from lethality induced by i.v. challenge with 10^6 CFU of an L. lactis strain expressing plasmid-encoded ClfA (34). Passive immunization with 1.5 mg of ClfA-specific monoclonal antibody 12-9 also protected mice against lethal L. lactis-ClfA challenge in mice. However, the Scully et al. studies do not support the efficacy of ClfA as a vaccine candidate against the pathogen S. aureus.

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ampicillin and arabinose as inducer. ClfA variants were extracted from the periplasm of E. coli cells using lysozyme. Samples were subjected to immobilized metal ion affinity chromatography by standard procedures, and fractions containing ClfA were pooled. Endotoxin was removed with a high-capacity endotoxin removal kit (Pierce), and ClfA was further purified by size exclusion chromatography in Tris-buffered saline, pH 7.4.

**IgG production and purification.** Purified rabbit IgGs were produced and provided by GlycoVaxyn AG, Schlieren, Switzerland. The rabbits were maintained, immunized, and bled at Eurogentec SA (Seraing, Belgium) according to institutional guidelines. New Zealand White rabbits were immunized subcutaneously on days 0, 7, 10, and 18 with 100 μg ClfAN123 or ClfAN23 mixed with Eurogentec’s proprietary adjuvant system according to their speedy protocol. The rabbits were euthanized on day 29, and sera from two rabbits each were pooled. IgG was purified from rabbits immunized with ClfAN123, ClfAN23, 2a-Epa, nontoxic alpha-hemolysin (Hla15/151), or S. aureus capsular polysaccharide type 8 (CP8)-Epa by protein A affinity chromatography as described previously (37).

**Bacterial strains and culture conditions.** Bacterial isolates used in this study included S. aureus strains LAC, ST80, Newman, Newman ΔclfA (strain DU5852), Newman Δspa (strain DU5873), and Reynolds (CP8). The mouse model of staphylococci cultivated for 24 h on Columbia agar (Difco) supplemented with 100 U/ml penicillin, 100 μg/ml of ClfAN123 or TBS in a total volume of serial threefold dilutions of rabbit IgG (3.33 to 0.013 μg/ml), mouse sera, or phosphate-buffered saline (PBS). The treated S. aureus cells were then added in duplicate to wells of a microtiter plate coated with 2 μg/ml human Fg (Calbiochem) and blocked with 5% skim milk. After incubation for 1 h at room temperature, the plates were washed and incubated for 15 min at 37°C with BacTiter-Glo lysing reagent (Promega), which produces a luminescent signal that is proportional to the amount of ATP present in the sample and corresponds to the number of adherent S. aureus. Luminescence was recorded on a SpectraMax L microplate reader (Molecular Devices, LLC), and the percent binding was calculated by dividing the signal of the antibody-treated S. aureus to that of PBS-treated control bacteria.

**OPK assay.** The HL60-based OPK assay was performed in 96-well plates as described previously (37). Each well (80 μl) contained 4 × 10⁵ viable and differentiated HL60 cells, 1 × 10⁵ CFU S. aureus, various dilutions of heat-inactivated mouse serum or rabbit IgG, and 0.1% guinea pig serum (Cedarlane) as a complement source. Control samples included S. aureus incubated with complement and HL60 cells (no antibody), S. aureus incubated with antibodies and complement (no HL60 cells), and S. aureus and HL60 cells only. The 96-well plates were incubated at 37°C with shaking at 700 rpm. After 2 h, 20 μl of 1% Triton X-100 was added to each well to lyse the HL60 cells. After a 3-min incubation at 37°C with shaking, 5-μl aliquots of the final reaction mixtures were plated in duplicate on tryptic soy agar plates. The percent change in the number of CFU/milliliter (i.e., killing) was defined as the reduction in the number of CFU/milliliter after 2 h compared with the number of CFU/milliliter at time zero. The opsonic titer was defined as the IgG concentration or serum dilution at which ≥50% of the inoculum was killed.

**S. aureus infection models.** Subcutaneous abscess formation was evaluated as described previously (21, 58). Briefly, groups of mice were anesthetized with ketamine and xylazine, and their backs were shaved and disinfected with 70% ethanol. An inoculum of 4 × 10⁵ S. aureus LAC was mixed with an equal volume of sterile dextran beads (Cytodex-1; Sigma), and 0.2 ml of the mixture was injected s.c. into the shaved flanks of immunized mice. After 48 h, the mice were euthanized, and the abscesses were aseptically excised and homogenized in 1 ml TSB. Serial dilutions of the homogenates were cultured quantitatively on tryptic soy agar plates (TSA) plates, and the results were expressed as the log CFU of S. aureus per abscess.

The mouse model of S. aureus surgical wound infection was previously described (59). Briefly, immunized mice were anesthetized with ketamine and xylazine, and their right thighs were shaved and disinfected. The thigh muscle was exposed, and a 1-cm incision was made in the muscle to the depth of the bone. The incision was closed with one silk (4-0 silk) suture, and 5 μl of an S. aureus suspension (90 to 300 CFU) was introduced into the muscle incision under the suture. The skin was closed with surgical wound clips. After 3 days, the mice were euthanized, and the muscle tissue was excised, weighed, and homogenized. Serial dilutions of the homogenates were plated in duplicate on TSA plates, and the results were expressed as the log CFU of S. aureus per gram of tissue.
For the lethality experiments, groups of 8 to 16 immunized mice were challenged i.v. with $1 \times 10^8$ CFU of S. aureus strain Newman or ST80. Survival was assessed for 14 days. For the sublethal renal abscess infection model (24, 60), mice were injected i.v. with 0.2 ml containing $2.5 \times 10^7$ to $1 \times 10^8$ CFU S. aureus ST80 or LAC. For certain experiments, the mice were bled by tail vein puncture 4 h after bacterial challenge for quantitative blood cultures. The mice were euthanized on day 3 and bled by cardiac puncture, and the kidneys and spleens were excised, weighed, and homogenized in 1 ml of TSB. Serial dilutions of the samples were plated in duplicate on solid medium, and the results were expressed as the number of CFU of S. aureus per milliliter of blood or per gram of tissue.

The bacteremia model was performed as described previously (37, 38). Briefly, immunized mice were inoculated by the intraperitoneal (i.p.) route with S. aureus in a 0.5-ml volume. Heparinized blood for culture was collected by tail vein puncture 1.5 h after bacterial challenge. Mouse weight loss and the bacterial burdens in the kidneys were quantified on day 4. Alternatively, mice were euthanized 1.5 h after i.p. challenge with S. aureus, and peritoneal lavage fluid samples (3 ml) were collected, serially diluted, and cultured quantitatively.

The endocarditis infection model was performed by methods similar to those we described previously (61). On day 0, male Sprague-Dawley rats (180 to 220 g; Charles River) were anesthetized with ketamine and xylazine. A polyethylene catheter was passed through the right carotid artery and the aortic valve until vigorous pulsation of blood within the catheter was observed. The catheter was sealed and tied in place, and the incision was closed. On day 1, rats with indwelling catheters were passively immunized by i.v. injection of 3 mg of IgG to ClfA or Shigella 2a-Epa. On day 2, a 0.2-ml inoculum containing $1.5 \times 10^8$ CFU S. aureus Newman was administered via the tail vein. On days 3, 4, and 5, heparinized blood was collected from each animal by tail vein puncture, and the number of CFU/milliliter of blood was determined by quantitative plating. Surviving rats were euthanized on day 5, and the aortic valve vegetations and kidneys were excised, weighed, and homogenized in TSB. Serial dilutions of the homogenates were plated in duplicate on TSA plates, and the results were expressed as the number of CFU of S. aureus per vegetation or per gram of tissue.

Statistical analysis. Statistical analyses were performed using Prism 6 (GraphPad Software, Inc.). P values were calculated using the Mann-Whitney test to compare median values of nonparametric data from two experimental groups. The Kruskal-Wallis test for nonparametric data was used to compare three or more unmatched groups, followed by the Dunn’s multiple-comparison posttest to compare the differences between each pair of groups. Survival rates in lethality experiments were analyzed by the log rank test. P values of <0.05 were considered significant.

SUPPLEMENTAL MATERIAL

Figure S1, TIF file, 0.1 MB.

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