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

Xue Li, a,b Xiaogang Wang, a Christopher D. Thompson, a Saeyoung Park, a,b Wan Beom Park, a,b,c Jean C. Lee a

Division of Infectious Diseases, Department of Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts, USA; Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China; Department of Internal Medicine, Seoul National University College of Medicine, Seoul, South Korea

* Present address: Saeyoung Park, Department of Medicine, University of Massachusetts Medical School, Worcester, Massachusetts, USA.

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.

Staphylococcus 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-acetylglucosamine 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 targeted 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. As shown in Fig. 1, the optimal dose for either ClfAN123 or ClfAN23 was 2 μg/mouse.

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.

RESULTS

ClfA immunogenicity. Mice were actively immunized with the N1, N2, and N3 subdomains of ClfA (ClfAN123) (aa 40 to 599) 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) as a control vaccine in murine models of septic arthritis (21), sepsis (20), and skin infection (21). 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 SAAAg 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 10^8 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.

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 SAAAg 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 10^8 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.

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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, inhibition of *S. aureus* Newman Δ espa mutant strain binding to immobilized Fg by rabbit anti-ClfAN23 IgG (α ClfA) (A) or mouse serum to ClfAN23 (B). Percent binding was calculated by dividing the signal of the antibody-treated *S. aureus* by the signal of untreated control bacteria.
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 ClfAN123 or ClfAN23. As shown in Fig. 4A, rabbit IgG raised to either ClfAN23 or ClfAN123 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 ClfAN23 and ClfAN123 IgG were not significantly different at the lower antibody concentrations. In contrast, rabbit IgG to ClfAN23 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<sup>+</sup> strains Reynolds (CP8) and ST80 (Fig. 4B) but not for the CP5<sup>+</sup> 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 ClfAN23 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 ClfAN23 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<sup>+</sup> 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 ClfAN123, ClfAN23, 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 adsorbed 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

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**FIG 5** 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 × 10⁸ 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** 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. 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 = 0.0048), 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

![Protective efficacy of ClfA in mice challenged i.v. with ~10^8 CFU of S. aureus Newman (8 mice/group) (A) or ST80 (8 to 16 mice/group) (B). Groups of mice were actively immunized with ClfAN23 or BSA mixed with alum plus SAS. Survival was monitored for 14 days, and the results were analyzed by the log rank test. Data in panel B are pooled from two independent experiments. **, P = 0.0048.](image1)

![Protective efficacy of ClfA in mice challenged i.v. with S. aureus ST80 (2.7 × 10^7 CFU/mouse) or LAC (1.3 × 10^8 CFU/mouse). Groups of mice were actively immunized with 2 μg of ClfAN23 or BSA mixed with alum plus SAS. On day 3 after challenge, the bacterial burdens in the kidneys (A and D) and spleens (B and E) and mouse weight loss (C and G) were quantified. Horizontal lines represent group medians, and the lower limit of detection by culture is depicted by dotted lines. The P value for the median values for the two groups in panel A, determined by the Mann-Whitney test, was 0.0648.](image2)
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 log_{10} CFU/ml) than mice given 2a-Epa antibodies (2.49 log_{10} CFU/ml), similar numbers of staphylococci were recovered from the peritoneal wash fluid samples (medians of 6.18 versus 5.81 log_{10} 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 × 10^8 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...
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.

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 CIFAN123 is readily degraded to ClfAN23 when expressed in *Escherichia coli* (49), CIFAN23 was utilized for the majority of our experiments. Only CIFAN23 has been crystallized for structural analysis (49), and its immunogenicity in mice was similar to that of CIFAN123 in our hands. We report that immunization with ClfA mixed with alum did not induce a Th1 or Th17 response in C57BL/6/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 CIFAN23 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 CIFAN23 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 CIFAN23, not in mice vaccinated with CIFAN123. In addition, we were able to achieve a significant increase in survival time in mice immunized with CIFAN23 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, catheter-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).

Fibrinogen, an acute-phase protein produced by the liver, is a major substrate for *S. aureus* during infection. For example, we observed that sera from immunized animals and bacteremic patients contained significantly higher levels of ClfA. We hypothesized that antibodies to ClfA (but not 2a-Epa) might block the fibrinogen-mediated aggregation of *S. aureus* within the peritoneal cavity, resulting in enhanced access of the bacteria to the bloodstream. To address this possibility, blood and peritoneal lavage cultures were performed on passively immunized mice challenged i.p. with strain LAC. Although the concentrations of *S. aureus* were significantly higher in blood samples 1.5 h after bacterial challenge, the peritoneal wash fluid samples from mice given 2a-Epa or ClfA antibodies yielded similar numbers of *S. aureus* (before or after sample sonication), suggesting that ClfA antibodies were not enhancing bacteremia by preventing bacterial aggregation within the peritoneal cavity. Moreover, we were not able to visualize differences in bacterial aggregation when peritoneal wash fluid samples were concentrated, stained, and visualized by confocal microscopy (data not shown). It is possible that ClfA antibodies prevented Fg-mediated clumping of *S. aureus* in the bloodstream itself, although the results of plating blood samples from infected mice before and after sonication did not support this hypothesis either (not shown). Nor did ClfA antibodies impact the bacterial burden in the spleens of mice challenged with either strain ST80 or LAC (Fig. 8). If opsonization with ClfA antibodies could promote phagocytosis of the unencapsulated strain LAC *in vivo* but not promote intracellular bacterial killing by the phagocytes, it is possible that these bacteria persist for longer periods in the bloodstream than nonopsonized *S. aureus*.

Our results differ from those of other groups who immunized with Freund’s adjuvant and showed that ClfA mediated protection against infection in mice. The protection against lethality (strain Newman) or enhancement of bacteremia (strain LAC) that we observed was strain dependent and affected by the challenge route of bacterial inoculation. We could not reproduce the findings of Narita et al. (24), who immunized BALB/c mice with ClfA adsorbed to alum and reported protection against infection of the kidneys and spleen following i.v. challenge. It is possible that differences in *S. aureus* challenge strains or inbred mouse strains could account for the discrepancies observed. Murphy et al. described the diversity of the ClfA protein sequence in a collection of clinical isolates (50). Our ClfA vaccine was derived from the strain Newman protein sequence, and Newman CIFAN123 is 91% identical (95% similar) to that produced by ST80 strains (51). However, the enhanced bacteremia that we observed in immunized mice was most striking following challenge with the USA300 strain LAC, which produces a ClfA protein with 99% identity in the N123 region to that of strain Newman (52). Thus, it is unlikely that the lack of efficacy that we observed can be attributed to sequence diversity in the ClfA protein, although Brady et al. demonstrated that the ClfA sequence type could affect ClfA immunogenicity and the quality of the antibody response (52).

By the very nature of rodent studies, they may have limited generalizability to human disease. Moreover, the correlates of protection against staphylococcal infection in humans remain unknown. Nonetheless, current vaccines that target microbial pathogens and are licensed for use in humans do protect mice against infection. Each antigen comprising a multicomponent vaccine should function to enhance protection consistently in at least one *S. aureus* infection model. The results of our studies do not support the inclusion of a ClfA antigen in a multicomponent *S. aureus* vaccine.

**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 CIFA P336Y338A (mutations that abrogate ClfA binding to fibrinogen [Fg]) [22]) comprising strain Newman N-terminal domains 1, 2, and 3 (CIFAN123) (aa 40 to 539) or N-terminal domains 2 and 3 (CIFAN23) (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 shaking flasks with super optimal or terrific broth medium with 100 μg/ml
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 (Hla(α13)), 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 ΔσA (strain DUS5852), Newman Δspa (strain DUS5873), and Reynolds (CP8) (9, 38, 44, 55). *S. aureus* was cultivated overnight in brain heart infusion broth (Difco) for use in fibrinogen binding inhibition studies or in tryptic soy broth (TSB) (Difco) for lethality studies. Opsonophagocytic killing (OPK) assays and bacteremia experiments were performed with staphylococci cultivated for 24 h on Columbia agar (Difco) supplemented with 2% NaCl (38). For all other infection models, *S. aureus* was harvested from TSB cultures grown to an optical density at 600 nm (OD600) of 0.8.

**Active and passive immunization studies.** Animal experiments were carried out in accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* (56), and the protocols were approved by the Harvard Medical School Standing Committee on Animals. Female C57BL/6 mice (4 weeks old; The Jackson Laboratory) were immunized on days 0, 14, and 28 by the s.c. route with ClfA adsorbed to 60 μg/ml of ClfAN23 or TBS in a total volume of 40 μl in 2% NaCl (38). For all other infection models, *S. aureus* was harvested from TSB cultures grown to an optical density at 600 nm (OD600) of 0.8.

**T cell assays and cytokine analyses.** To define the immune cell populations induced by vaccination, C57BL/6 animals were immunized s.c. as described above with ClfAN23 adsorbed to alum or adsorbed to alum and mixed with SAS. Control mice were given Tris-buffered saline (TBS) mixed with alum or alum plus SAS. One week after the last immunization, the cervical, axillary, and brachial lymph nodes were harvested from each animal, and the cells were passed through 100-μm mesh nylon screens. Cells (2 × 10⁶ cells) were seeded in wells of a 24-well tissue culture plate and stimulated with either 10 μg/ml of ClfAN23 or TBS in a total volume of 1 ml RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM 1-glutamine, 10% fetal bovine serum, and 50 μM 2-mercaptoethanol. After 6 days in culture, the supernatants were collected and analyzed by ELISA (R&D system) to measure interleukin 17 (IL-17), interferon gamma (IFN-γ), and interleukin 4 (IL-4) production. The lymph node cell cultures were incubated for an additional 6 h with 30 ng/ml phorbol myristate 1-ace-tate, 1 μg/ml ionomycin, and 1 μl of BD GolgiPlug (BD Biosciences) prior to cell harvest. The cells were washed with staining buffer (0.01 M phosphate buffer, 1% fetal bovine serum, and 0.09% sodium azide) and incubated with fluorescein isothiocyanate (FITC)-labeled anti-CD4 (rat IgG2a; BioLegend) or isotype control antibodies. The cells were then treated with fixation/permeabilization solution (BD Biosciences) and stained with rat phycoerythrin (PE)-labeled anti-mouse IL-17A, allophycocyanin (APC)-labeled anti-IFN-γ, or their isotype control antibodies (BioLegend). Cells gated on CD4 were analyzed by flow cytometry to assess the frequency of Th1 (CD4+ IFN-γ+ IL-17−) or Th17 (CD4+ IL-17+ IFN-γ−) lymphocytes induced by vaccination compared to the frequencies in mice given adjuvant only. The samples were analyzed on a BD FACScan instrument, the data were analyzed using CellQuest software, and the results were expressed as the fold change in percentage of cytokine-producing T cells compared to the cells from control mice.

**Fg binding inhibition assay.** The ability of mouse serum or rabbit IgG to inhibit *S. aureus* binding to immobilized Fg was measured by a previously described assay (57) with slight modifications. Briefly, 5 × 10⁵ CFU of strain Newman Δspa cells in 60 μl was incubated for 1 h at room temperature with an equal 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 BactTiter Gly lysis 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 × 10⁶ CFU of *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. 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 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 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 × 10⁷ 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 × 10⁷ to 1 × 10⁸ 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, seri- ally 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 CRM197. On day 2, a 0.2-ml inoculum containing ~1.5 × 10⁷ 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/ml/litter 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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