Vaccine composition formulated with a novel TLR7-dependent adjuvant induces high and broad protection against *Staphylococcus aureus*

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Both active and passive immunization strategies against *Staphylococcus aureus* have thus far failed to show efficacy in humans. With the attempt to develop an effective *S. aureus* vaccine, we selected five conserved antigens known to have different roles in *S. aureus* pathogenesis. They include the secreted factors α-hemolysin (Hla), ess extracellular A (EsxA), and ess extracellular B (EsxB) and the two surface proteins ferric hydroxamate uptake D2 and conserved staphylococcal antigen 1A. The combined vaccine antigens formulated with aluminum hydroxide induced antibodies with opsonophagocytic and functional activities and provided consistent protection in four mouse models when challenged with a panel of epidemiologically relevant *S. aureus* strains. The importance of antibodies in protection was demonstrated by passive transfer experiments. Furthermore, when formulated with a toll-like receptor 7-dependent (TLR7) agonist recently designed and developed in our laboratories (SMIP.7–10) adsorbed to alum, the five antigens provided close to 100% protection against four different staphylococcal strains. The new formulation induced not only high antibody titers but also a Th1 skewed immune response as judged by antibody isotype and cytokine profiles. In addition, low frequencies of IL-17–secreting T cells were also observed. Altogether, our data demonstrate that the rational selection of mixtures of conserved antigens combined with Th1/Th17 adjuvants can lead to promising vaccine formulations against *S. aureus*.

Staphylococcus aureus | vaccine | TLR7 | adjuvant | Hla

Current antibiotics are not efficacious against emerging multidrug-resistant strains of *Staphylococcus aureus*, a major human pathogen. Therefore, there is an urgent need to develop vaccines to target this pathogen. Two prophylactic vaccines have been tested recently for efficacy in humans: StaphVAX, which contained capsular polysaccharides type 5 and 8 (CP5 and CP8), and V710, based on a single protein antigen (IsdB) (1, 2). Both vaccines failed in phase III efficacy trials (3, 4). On the basis of these disappointing results and taking into account that *S. aureus* produces a plethora of virulence and immune evasion factors, different vaccine candidates, constituted by multiple components, are currently in phase I/II trials, but efficacy data are not available yet (5). In line with the multicomponent strategy, our laboratory has undertaken a vaccine discovery project aiming at the identification of conserved antigens, which play important roles in *S. aureus* virulence and pathogenicity. The main objective of the study was to combine the selected antigens in the presence of appropriate adjuvants and to demonstrate protective efficacy against a panel of genetically different *S. aureus* clinical isolates in different mouse models.

Results

Antigen Selection. The antigens included in our candidate combination vaccine were selected among surface and secreted factors previously shown to be protective and involved in *S. aureus* virulence. Two of them, the ferric hydroxamate-binding lipoprotein FhuD2 and the putative lipoprotein named conserved staphylococcal antigen 1A (Csa1A), are surface-exposed antigens that were identified in our laboratories using MS-based surfome analyses and bioinformatics (6, 7). FhuD2 is a lipoprotein involved in iron uptake and in early stages of invasive *S. aureus* infection (6, 8, 9). Csa1A is highly conserved across different *S. aureus* isolates (Fig. 1*A*) and belongs to a family of proteins encoded in at least four distinct loci sharing from 54% to 91% sequence identity (7). The other three selected antigens are secreted virulence factors and include α-hemolysin (Hla), ess extracellular A (EsxA), and ess extracellular B (EsxB). Hla is one of the best characterized toxins of *S. aureus* and has been shown to play a prominent role in early stages of

Significance

***Staphylococcus aureus* is a human pathogen causing life-threatening infections. The high incidence of methicillin-resistant *S. aureus* isolates resistant to all antibiotics makes the development of anti-*S. aureus* vaccines an urgent medical need. However, the unique ability of *S. aureus* to produce virulent factors, which counteract virtually all pathways of innate and adaptive immunity, has hampered all vaccine discovery efforts. Starting from the assumption that to be effective a vaccine should induce highly functional antibodies and potentiate the killing capacity of phagocytic cells, we selected a cocktail of five conserved antigens involved in different mechanisms of pathogenesis, and we formulated them with a potent adjuvant. This vaccine provides an unprecedented protective efficacy against *S. aureus* infection in animal models.**

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A

	EsxA	EsxB	Hla	FhuD2	Csa1A
number of strains coding a full length protein (%)	109/109 (100)	69/109 (63)	98/109 (90)	109/109 (100)	60/60
% protein identity	≥ 99%	≥ 96%	≥ 97%	≥ 99%	≥ 83%

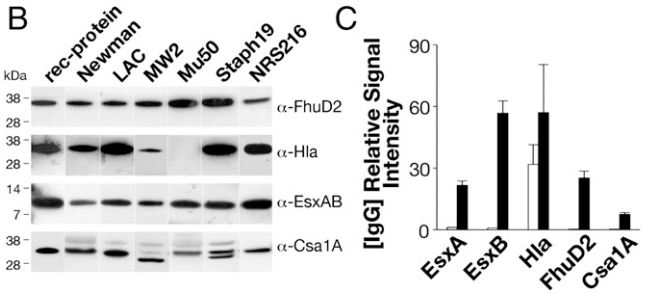

Fig. 1. Gene conservation and expression of *EsxA/B, Hla, FhuD2, and Csa1A*. (*A*) Conservation of *esxA/B, hla, fhuD2, and csa1A* genes among representative *S. aureus* strains. Conservation data of *csa1A* are limited to 60 staphylococcal strains with genomes available in databases. (*B*) In vitro expression of 4C-Staph antigens was assessed by Western blotting in the indicated strains. See Tables S1–S3 for further information about the strains. Sera raised against the vaccine antigens recognized the proteins expressed by *S. aureus* at the expected molecular weight. (*C*) *S. aureus*-infected mice have antibodies against the vaccine antigens. Sera from BALB/c mice ($n = 15$) immunized with SpAKKAA (closed bars) or with PBS/adjuvant control (open bars) and then challenged by i.v. inoculation with 5×10^6 CFU *S. aureus* USA300 LAC were analyzed for antibody responses to staphylococcal antigens by dot-blot analysis. Data represent the mean ± SEM and are representative of three independent experiments.

invasive and skin infections in animal models (10, 11). EsxA and EsxB are two factors secreted through the ESAT-6 secretion system (ESS) of *S. aureus* associated with abscess formation and may facilitate persistence and spread of the pathogen in the infected host (12, 13). To be exploited as vaccine components, Hla, EsxA, and EsxB were modified as follows. Hla was detoxified by a histidine to leucine substitution at position 35 (Hla_{H35L}), resulting in a mutated protein unable to form pores and lyse host cells (see Fig. 4*C*) and to cause cell junction dissolution (14). EsxA and EsxB were fused together, creating a recombinant 24-kDa EsxAB chimera, which was stable and well expressed in *Escherichia coli* unlike the individual proteins. Therefore, we named the combination vaccine 4C-Staph (four-component *S. aureus* vaccine).

Selected Vaccine Antigens Are Conserved and Expressed in Vivo. The presence and conservation of *fhuD2, csa1A, hla, esxA*, and *esxB* genes in *S. aureus* were investigated by analyzing 60 genome sequences available in public databases, as well as by sequencing the genes from 49 isolates of our internal strain collection (Tables S2 and S3). As reported in Fig. 1*A*, *fhuD2, csa1A,* and *esxA* are present in all strains and highly conserved (amino acid identity ranging from 83% to 99%). In general, *hla* is also well conserved, but the sequence contains premature stop codons in 11 strains belonging to clonal complex 30 (CC30) of the 26 used in this study. Finally, *esx*B is missing in 37% of the analyzed strains, although most of these strains belong to the same clonal complex (CC30). However, when present, EsxB protein is highly conserved. Next, we investigated the expression of the antigens in six epidemiologically relevant *S. aureus* strains that were also used in mouse models of *S. aureus* infection (Fig. 1*B* and Table S2). Expression analysis was carried out by Western blot on cell extracts and culture supernatants. As shown in Fig. 1*B*, FhuD2 and EsxA/EsxB were expressed in all six strains. However, because EsxA and EsxB comigrated in the gel, we could not unambiguously confirm that the strains simultaneously expressed both proteins. Furthermore, all strains but Mu50 expressed Hla. This result was unexpected because the sequence analysis of the *hla* gene in the Mu50 strain did not reveal frameshifts or premature stop codons. Finally, Csa1A was expressed in Newman, Los Angeles County clone (LAC), Mu50, and NRS216. In Staph19, in addition to the immune reactive band corresponding to Csa1A, a second band migrating at a slightly lower molecular mass was present. A similar immune reactive protein was detected in MW2 where Csa1A was poorly or not expressed under the conditions used. As already pointed out, Csa1A belongs to a family of homologous proteins encoded by different loci in *S. aureus* genome and the number of homologs varies in the different strains. Therefore, it is not surprising that different immune reactive protein profiles are visible in the total extracts of different *S. aureus* isolates. To understand if the antigens included in the vaccine are expressed and immunogenic during invasive infection, we analyzed sera of mice infected with the *S. aureus* LAC strain for their reactivity against the purified vaccine proteins. Of the four vaccine components, only Hla had a detectable immunoreactivity in a dot-blot assay (Fig. 1*C*). We hypothesized that the lack of reactivity against the other vaccine components was due to the expression of the staphylococcal protein A (SpA) by *S. aureus* during infection. Indeed, SpA is a B-cell superantigen: interaction with B-cell receptors can induce clonal expansion and subsequent apoptosis of B1 and MZ B cells dampening the humoral response against staphylococcal antigens (15). Therefore, the elicitation of vaccine antigen-specific antibodies could be prevented by SpA through deletion of antigen-specific B cells. To test this hypothesis, we decided to use a nontoxigenic form of SpA (SpA_{KKAA}), developed previously by Kim et al. (16), to immunize animals before their infection. By doing so, we inhibited the activity of SpA as previously demonstrated (16). As expected, sera of mice immunized with SpA_{KKAA} and then infected with *S. aureus* recognized all of the vaccine components (Fig. 1*C*).

Combination of Hla_{H35L}, EsxAB, FhuD2, and Csa1A Induces Consistent Protective Immunity Against Epidemiologically Relevant Staphylococcal Strains in Different Mouse Models. One of the criteria for selecting the four antigens was their ability to induce partial protection in one or more mouse models of *S. aureus* infection. We then asked the question of whether, when combined in a single formulation, the antigens could work synergistically and induce broad protection. Mice were immunized with each antigen alone or with the combination of the four antigens formulated with aluminum hydroxide (4C-Staph). After immunization, mice were challenged i.v. with a sublethal dose of one of the five strains, Newman, LAC, Mu50, Staph19, and MW2, and 4 d later, bacteria recovered from kidney homogenates were counted. As shown in Table 1, immunization with 4C-Staph resulted in a statistically significant reduction of bacterial load regardless of the challenge strain used compared with control mice immunized with alum alone (Alum). Reduction of colony forming units (CFUs) varied from a minimum of 1.37 to a maximum of 2.44 logs. Immunization with single vaccine components and IsdB did not provide such a consistent protection, and it was generally inferior to that provided by 4C-Staph (Table S4). IsdB, an *S. aureus*-conserved surface antigen shown to be protective in mice and tested in clinical trials (1, 2), was used as comparator throughout these experiments. The most effective single antigen in this model was FhuD2, which protected against four of five strains with a reduction in CFUs ranging from 0.94 to 2.13 logs. CFU counts measured following immunization with 4C-Staph and single antigens were closely paralleled by reduction of the area and number of abscesses (Fig. S1 and Table S5). We also compared the performance of 4C-Staph with respect to single antigens in the peritonitis infection model by challenging mice with a lethal dose of either Newman or MW2 strain. None of the single antigens significantly increased survival rate of mice challenged with the strain Newman (Table 2), and only HlaH35L,

Table 1. Summary of 4C-Staph efficacy in reducing bacterial load in the renal abscess model

Immunization	Challenge strain and dose (CFU/mouse)	N	Mean log CFU ± SE	Log CFU reduction*	P
Alum	ST254 (Newman)	16	7.53 ± 0.30		
4C-Staph	2.0×10^7	16	5.96 ± 0.36	1.57	**0.002**
Alum	USA300 (LAC)	20	7.01 ± 0.18		
4C-Staph	3.8×10^7	19	4.57 ± 0.49	2.44	**<0.0001**
Alum	USA400 (MW2)	16	7.11 ± 0.24		
4C-Staph	2.9×10^7	20	4.80 ± 0.50	2.31	**0.001**
Alum	USA100 (Mu50)	19	7.49 ± 0.17		
4C-Staph	4.2×10^7	19	5.98 ± 0.39	1.51	**0.003**
Alum	ST80 (Staph19)	18	7.43 ± 0.19		
4C-Staph	4.9×10^7	20	5.20 ± 0.33	2.23	**<0.0001**

Mice were immunized with 4C-Staph or with alum alone and then challenged as indicated (at least two separate experiments). One-tailed Mann-Whitney *u* test. Values of $P < 0.01$ are highlighted in bold. Complete results including single antigens and IsdB are reported in Table S2.
*Log CFU reduction = mean log CFU alum ctrl − mean log CFU vaccinated.

Csa1A, and FhuD2 significantly increased the median survival time of the mice (Table S6). On the other hand, vaccination with Csa1A, EsxAB, and FhuD2, but not with HlaH35L and IsdB, significantly increased the number of surviving mice challenged with strain MW2 (Table 2). Csa1A and FhuD2 also significantly increased the median survival time of mice infected with the strain MW2 (Table S6). Overall, 4C-Staph induced the greatest level of protection against both strains (Table 2 and Table S6). The protective efficacy of 4C-Staph was further investigated with three additional types of experiments. First, we expanded the peritonitis protection data by challenging the vaccinated mice with four additional strains (LAC, Staph19, Mu50, and NRS216), and we followed mouse survival over a period of 15 d. As shown in Fig. 2*A*, the 15-d protection elicited by 4C-Staph ranged from 53% to 78% and was always significantly superior to that observed in mock (for all strains) and IsdB-immunized animals (for strains Newman, LAC, MW2, and Staph19). IsdB immunization was also tested in independent experiments against strains NRS216 and Mu50 (Fig. S2). As observed against the other staphylococcal strains, IsdB immunization did not confer significant protection against these latter two strains. Second, we tested the protective efficacy of 4C-Staph in the pneumonia model, challenging mice with five different strains: Newman, LAC, Staph19, Mu50, and NRS216. In this model, survival rate of mice immunized with 4C-Staph ranged between 87% and 47%, with the exception of strain Mu50 (Fig. 2*B*). However, this was due to the low mortality rate associated with this strain in the pneumonia model (23% mortality only in the control group). For the same reason strain MW2 was not included in these experiments. This is consistent with the fact that Hla has been shown to play a predominant role in the pneumonia model (11) and that its expression is low or nondetectable in strains MW2 and Mu50, respectively (Fig. 1*B*). IsdB never conferred significant protection in this model. Finally, to further assess the protective efficacy of 4C-Staph against different staphylococcal disease outcomes, we used a skin infection model. In this model, mice were inoculated by s.c. injection in the shaved right flank with *S. aureus* LAC strain. Mouse skin and abscesses were harvested on day 4 after inoculation for CFU enumeration and histopathology. In a different set of animals, abscess mass and dermonecrotic area were monitored at 24-h intervals for 14 d. Immunization with 4C-Staph significantly reduced abscess formation and CFU counts (Fig. 3 *A* and *B*). Furthermore, as shown in the gross histology pictures and the H&E-stained tissue sections, dermonecrosis was substantially absent in the vaccinated mice (Fig. 3 *C*–*F*).

Protection Induced by 4C-Staph Was Largely Mediated by the Elicitation of Functional Antibodies. To dissect the possible mechanisms of protection induced by the vaccine, we focused our attention on the analysis of functional antibodies. To this aim, rabbits were immunized with 4C-Staph, and subsequently the rabbit serum was used in passive protection experiments. In particular, 150 μL of serum were administered i.v. to mice, and 24 h later animals were challenged with *S. aureus* Newman strain according to the abscess and peritonitis models. As shown in Fig. 4*A*, CFU counts in the kidneys of animals that received the hyperimmune serum were significantly lower than in control animals. Likewise, survival of passively immunized mice to the i.p. *S. aureus* challenge was significantly greater than that of the control group (58% vs. 19%; Fig. 4*B*). On the other hand, rabbit serum against IsdB did not provide significant protection against neither abscess formation nor in the peritonitis model (Fig. 4 *A* and *B*). We next investigated the mechanisms by which 4C-Staph antibodies protected mice against *S. aureus* challenge. First, we tested anti-Hla neutralizing activity of the rabbit serum used in the passive protection experiment. To this end, rabbit erythrocytes were incubated for 30 min with 50 nM recombinant WT Hla together with different concentrations of rabbit serum. As shown in Fig. 4*C*, Hla-mediated hemolysis was inhibited in a dose-dependent manner, with 0.5% serum concentration sufficient to mediate close to complete inhibition. No inhibition was mediated by high concentrations (8%) of control serum. Furthermore, we analyzed the ability of the 4C-Staph mouse antiserum to promote opsonophagocytosis of *S. aureus* Newman by differentiated HL60 cells in the presence of rabbit complement. As shown in Fig. 4*D*, the serum from animals immunized with 4C-Staph mediated ∼40% killing of bacteria within 1 h of incubation. Preimmune serum and serum from mock-immunized animals did not promote bacterial killing. The same was true when 4C-Staph rabbit antiserum was tested in the absence of phagocytes or active complement.

The Protective Efficacy of 4C-Staph Was Further Improved when Formulated with a Novel Small Molecule Immune Potentiator. It is likely that an efficacious *S. aureus* vaccine would induce both functional antibodies and $CD4^+$ T cell-mediated immunity skewed toward a T helper (Th) type 1/Th17 response (4, 17–20). We therefore hypothesized that if 4C-Staph was formulated with

Table 2. Summary of 4C-Staph efficacy in increasing survival rates in the peritonitis model

Immunization	Challenge strain and dose	N	Percent survival	P vs. alum*	P vs. 4C-Staph*
Alum	Newman	32	22		**0.001**
Csa1A	5.5×10^8	32	31	0.29	0.01
EsxAB		32	38	0.14	0.04
HlaH35L		32	31	0.29	0.01
FhuD2		32	38	0.14	0.04
4C-Staph		32	63	**0.001**	
IsdB		32	38	0.14	0.04
Alum	MW2	60	30		**0.0001**
Csa1A	8×10^8	60	60	**0.0008**	0.087
EsxAB		60	48	0.03	**0.004**
HlaH35L		60	43	0.09	**0.0008**
FhuD2		60	52	0.01	0.01
4C-Staph		60	61	**0.0001**	
IsdB		59	44	0.08	**0.001**

Mice were immunized with the indicated antigens or with alum alone and then challenged with the indicated strains (at least three separate experiments).
*Fisher's exact test. Values of $P < 0.01$ are highlighted in bold. Complete results are reported in Table S6.

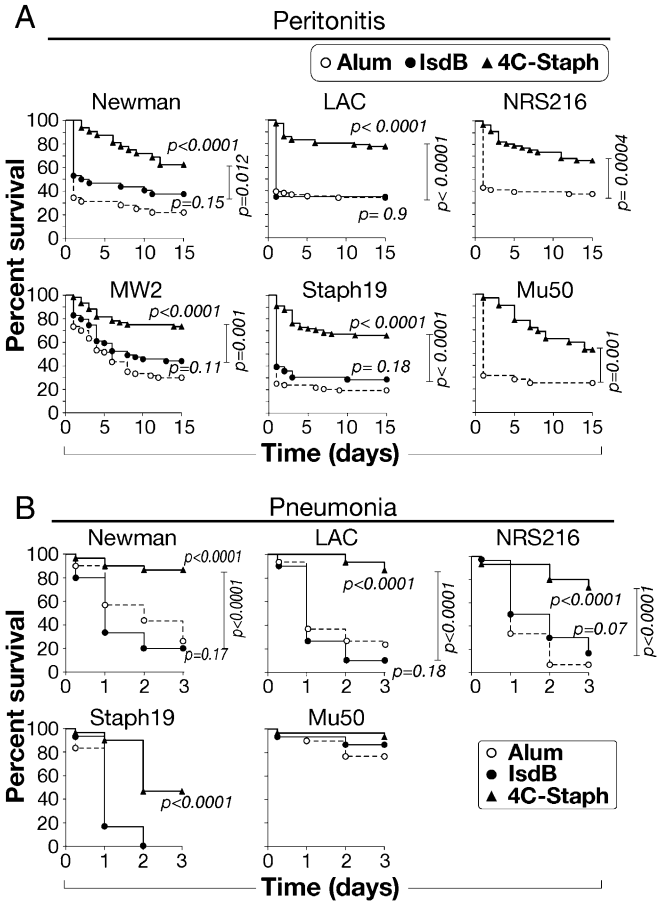

Fig. 2. 4C-Staph generates greater protection against clinically relevant *S. aureus* strains than IsdB in the peritonitis and pneumonia models. In all plots, lines with triangles indicate 4C-Staph; lines with closed circles indicate IsdB; and lines with open circles indicate mice treated with aluminum hydroxide alone. (*A*) In the peritonitis model, mice were challenged with the strains Newman, LAC, MW2, Mu50, Staph-19, and NRS216, respectively (n = 32–88 per group, at least two separate experiments). (*B*) In the pneumonia model, mice were challenged with the strains Newman, LAC, Staph-19, and NRS216, respectively (n = 30 per group, two separate experiments). Statistical analysis was performed by log-rank (Mantel-Cox) test.

an adjuvant stimulating this kind of immune response, its protective properties might be further enhanced. In the course of our internal adjuvant discovery program, an engineered small molecule immune potentiator (SMIP.7–10) targeting Toll-like receptor 7 (TLR7) was generated (21). Such an immune potentiator has been chemically functionalized to be efficiently adsorbed to aluminum hydroxide, and the resulting adjuvant was named SMIP.7–10-Al$(OH)_3$. We tested the vaccine combination formulated with SMIP.7–10-Al$(OH)_3$ (4CT7-Staph) in the peritonitis model, and protection was compared with that achieved by 4C-Staph (formulated with alum). Ten days after vaccination, mice were challenged with a lethal dose of Mu50, Newman, LAC, and MW2 strains, and survival was followed over a period of 15 d. As shown in Fig. 5*A*, 4CT7-Staph was highly efficacious against all strains, with protection levels ranging from 80% to 90% and significantly higher than that obtained when the antigen combination was formulated in alum alone. Protection correlated with an increase in total IgG titers against all antigens except Hla (Fig. 5*B*). We next analyzed CD4 T-cell responses against the combined antigens induced by 4CT7-Staph or 4C-Staph immunization. To this aim, we performed two types of experiments. First, we looked at the isotype profile of antigen-specific IgGs induced by 4C-Staph and 4CT7-Staph. As shown in Fig. 5*B*, a significant increase in antigen-specific IgG2a was observed in animals vaccinated in the presence of SMIP.7–10. An increase was also observed for IgG2b and IgG1, albeit not for all antigens. Second, we analyzed antigen-specific CD4 T-cell responses induced by the vaccine formulated with the two adjuvants. For this purpose, we measured intracellular cytokines produced by $CD4^+CD44^{high}$ T cells from spleens of mice immunized with the two formulations in response to in vitro stimulation with the combined vaccine proteins or OVA as a negative control. As shown in Fig. 5*C*, both formulations induced statistically higher frequencies of IL-2–, TNF-, and IFN-γ–producing $CD4^+CD44^{high}$ T cells than those induced by the respective adjuvant alone, whereas only 4C-Staph induced also IL-4/IL-13–producing cells. Greater frequencies of IL-17^+ cells were observed in mice vaccinated with 4CT7-Staph, although differences were not statistically significant.

Discussion

Among all bacterial vaccines still missing, *S. aureus* is probably one of the most difficult to develop for a number of reasons. First, there is no clear evidence of natural protective immunity against *S. aureus,* and there are no correlates of protection established yet. Second, *S. aureus* expresses a plethora of toxins and immune evasion factors (22, 23). Third, a large proportion of at-risk population includes immune-compromised subjects (e.g., HIV, cancer and hemodialysis patients, as well as the elderly). Taking into account these considerations, our strategy to develop a *S. aureus* vaccine was (*i*) to select antigens with different roles in pathogenesis, (*ii*) to increase the reliability and predictive value of this study by using different mouse models and functional assays, (*iii*) to include an adjuvant that could elicit high antibody titers and potentiate vaccine efficacy.

The antigens of 4C-Staph were selected based on the hypothesis that they could work synergistically by eliciting antibodies with different mechanisms of action. On one hand, we

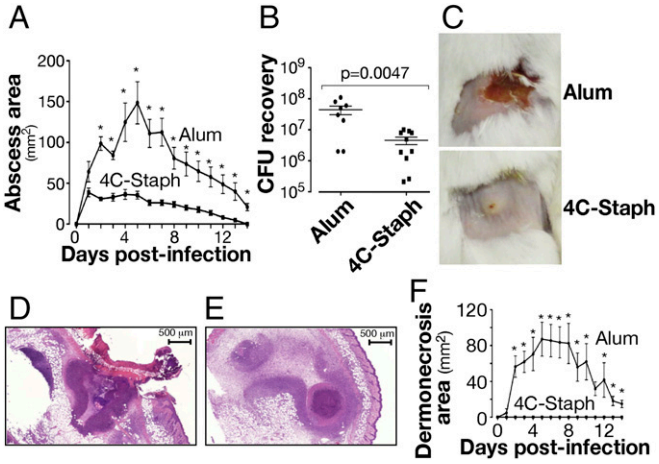

Fig. 3. Reduction of abscess formation and dermonecrosis following immunization with 4C-Staph. Active immunization with 4C-Staph decreases size of abscesses (*A*) and CFU counts (*B*) associated with *S. aureus* LAC infection. Abscess formation was monitored once per day for 2 wk (*A*) or 4 d after s.c. infection with 1×10^7 of LAC 10 d after secondary immunization (*B*). Results are the mean ± SEM and N = 10 mice per group. (*C*) Representative mouse skin lesions (day 5). (*D* and *E*) Representative histological sections showing mouse LAC abscess with dermonecrosis (at 3 d after infection) following immunization with either alum alone (*D*) or 4C-Staph (*E*). Magnification of images is ×200. (*F*) Dermonecrotic area monitored once per day for 2 wk. Results are the mean ± SEM and N = 10 mice per group. Data were analyzed using a one-way ANOVA and Dunnett's test. $^*P < 0.05$.

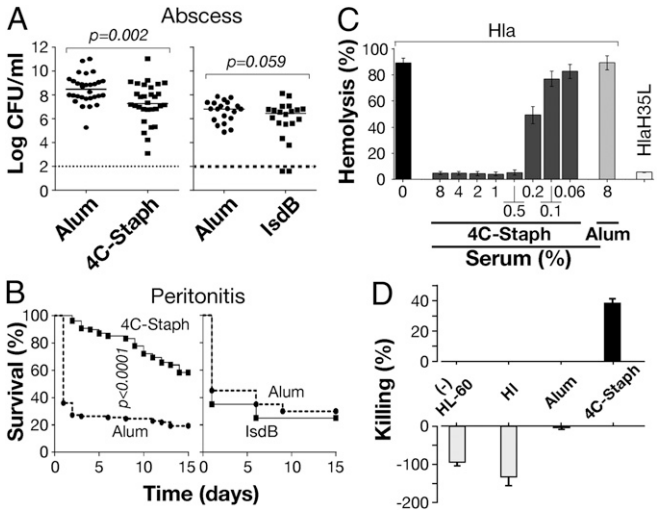

Fig. 4. 4C-Staph generates functional antibodies. (*A* and *B*) Mice received i.v. sera of rabbits immunized with alum alone, 4C-Staph, or IsdB, and 24 h later animals were infected i.v. (abscess model) or i.p. (peritonitis model) with *S. aureus* Newman. In the abscess model, the dashed line indicates the lower limit of CFU detection ($n > 20$ per group, at least two separate experiments). Statistical analysis was performed by log-rank (Mantel-Cox) test for the peritonitis model and Mann–Whitney *U* test for the abscess model. (*C*) Rabbit antisera against the 4C-Staph vaccine neutralize Hla hemolytic activity. The black and gray columns represent hemolysis obtained incubating rabbit erythrocytes with 50 nM Hla without rabbit serum or with serial dilutions of a serum from a rabbit immunized with 4C-Staph or Alum alone, as indicated in the figure. The white column corresponds to the hemolysis observed with 50 nM Hla_{H35L}. Columns represent mean value ± SD of four independent rabbit sera. (*D*) Sera against 4C-Staph mediate *S. aureus* opsonophagocytosis. Mouse vaccine antisera, rabbit complement, HL-60, and the *S. aureus* strain Newman were incubated for 1 h and plated on tryptic soy agar for CFU counting. Percent killing was calculated as the ratio between the percent killing achieved with and without sera. No bacterial killing was observed in the presence of sera from alum-treated mice (Alum) or using heat-inactivated complement (HI) and in absence of HL-60 cells [(–) HL-60]. Error bars represent SD. Statistical analysis was performed by paired *t* test.

showed that our vaccine induces antibodies that target three secreted virulence factors: the pore-forming toxin Hla, known to play key roles in pneumonia and skin infection (10, 11), and EsxA/EsxB, involved in abscess formation (12). In addition, because of the presence of the surface-associated lipoproteins FhuD2 and Csa1A, our vaccine elicits antibodies that are not only opsonophagocytic but also interfere with important biological functions. In the case of anti-FhuD2 antibodies, they can impair iron uptake (6, 9), whereas as far as anti-Csa1A antibodies are concerned, we are accumulating evidence that they could inhibit cell wall biosynthesis, a process that Csa1A is likely to participate in. Our sequence analysis revealed that the five selected antigens are conserved in most *S. aureus* isolates. In addition of being conserved, the antigens were found expressed under laboratory conditions and in experimentally infected mice. In vivo expression was deduced by detecting antigen-specific antibodies in mice infected with sublethal doses of *S. aureus*. Interestingly, as previously reported (16), these antibodies were found only if infected mice had preexisting SpA antibodies, which could neutralize the capacity of SpA to induce B-cell apoptosis. By analyzing sera from healthy adult volunteers, we found that with the exception of Hla, antibodies against the vaccine antigens were present in few individuals (Fig. S3). Considering the high frequency with which humans are exposed to *S. aureus*, it is plausible to believe that the low immunogenicity of the vaccine antigens is not due to their poor expression but rather to the same antibody inhibitory activity exerted by SpA in mice. This observation might have important implications for the strategy to be used for vaccine candidate identification. A frequently used approach to vaccine antigen selection is the analysis of sera from convalescent patients. Although this approach has been successful for a number of pathogens, *S. aureus* might represent an important exception. When formulated in alum, the vaccine antigens induced robust protection in four different mouse models of infection. Importantly, protection was achieved when mice were challenged with six genetically different *S. aureus* strains, isolated from human patients and belonging to important

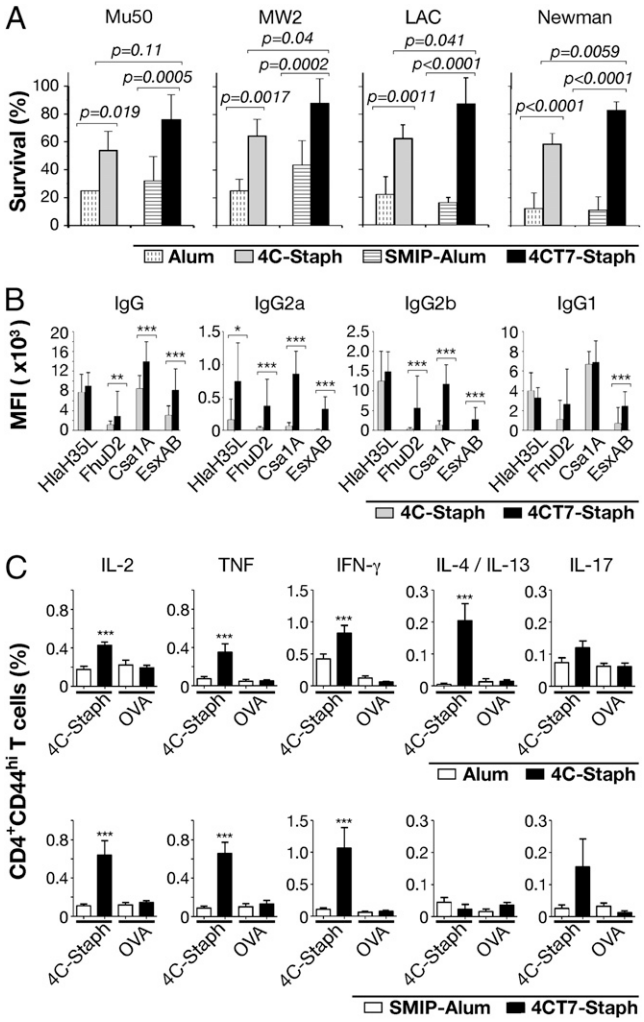

Fig. 5. Protective efficacy, isotype profile of vaccine-specific IgGs, and $CD4^+$ T-cell responses induced by 4C-Staph and 4CT7-Staph. (*A*) Protective efficacy of 4C-Staph and 4CT7-Staph in the peritonitis model against *S. aureus* strains Mu50, MW2, LAC, and Newman. In all graphs, white columns indicate the adjuvants alone [SMIP.7–10-$Al(OH)_3$ = SMIP-Alum]. Gray and black columns, one of the two vaccine formulations as indicated (n = 32–64 per group, at least two separate experiments). (*B*) Antigen-specific IgGs in mice immunized with 4C-Staph or 4CT7-Staph. (*C*) $CD4^+CD44^{high}$ T-cell responses in immunized mice. Graphs show results from mice immunized with adjuvant alone (white bars, Alum or SMIP-Alum) or with vaccine antigens formulated with the two adjuvants (black bars, 4C-Staph or 4CT7-Staph). Splenocytes collected from each mouse and stimulated in vitro with the vaccine antigens or OVA, used as a control. The graphs show the frequencies of $CD3^+CD4^+$ $CD44^{high}$ T cells expressing IL-2, TNF, IFN-γ, IL-4, and/or IL-13, and IL-17A. Data are expressed as mean ± SE from individual mice (n = 7) and are representative of two independent experiments. Statistical significance of mice immunized with vaccine + adjuvant vs. adjuvant only (***$P \leq 0.001$).

epidemic lineages. None of the single antigens performed similarly well, suggesting that each antigen contributed at least to some extent to protection induced by 4C-Staph. Immunization with IsdB, which was used as comparator throughout this study, elicited nonconsistent protection against the *S. aureus* strains tested in the kidney abscess model. In addition, in the peritonitis and pneumonia models, IsdB did not confer significant protection and was significantly inferior to the 4C-Staph. Protective immunity was largely mediated by antibodies, as indicated by the fact that similar levels of protection were obtained when mice were passively immunized with sera from rabbits vaccinated with 4C-Staph. Although antibodies are expected to have an important role in preventing *S. aureus* human infections, the need to efficiently kill the pathogen once it gets internalized into phagocytic cells suggests that the addition of a Th1/Th17 adjuvant could be beneficial in eliciting optimal anti-*S. aureus* immune responses. Indeed, a Th1/Th17 polarized immune response has been demonstrated to potentiate the opsonophagocytic activity of neutrophils and macrophages (17–19). We tested this hypothesis by formulating the vaccine antigens with SMIP.7–10-Al $(OH)_3$, which is a small molecule TLR7 agonist chemically functionalized to allow stable adsorption to aluminum hydroxide. This feature prevents side effects due to systemic exposure of the SMIP and allows the codelivery of the antigen-SMIP complex to antigen presenting cells, a prerequisite for optimal activation of antigen-specific B and T cells (21). 4CT7-Staph was found highly protective in the mouse models used, with almost 100% of mice surviving lethal doses of different *S. aureus* isolates. The presence of the SMIP not only enhanced the antibody titers against vaccine antigens but also skewed the immune response toward a Th1/Th17 profile, as judged by antibody isotype and cytokine profiles. Therefore, the increased efficacy associated with SMIP.7–10-Al $(OH)_3$ compared with the alum formulation may likely be due to its combined effects on antibody and T-cell responses.

Two prophylactic vaccine trials have recently failed, and in both cases, the vaccines did not include adjuvants. In particular, the most recent phase III trial (3) was carried out with a single dose of IsdB with no adjuvants. Our data seem to indicate that part of the failure might be due to an inefficient stimulation of cell-mediated immunity and prompt to test the use of new vaccines able to activate this arm of the immune system, which appears to be particularly important to combat *S. aureus* infections.

Materials and Methods

Vaccine antigens were cloned from the *S. aureus* NCTC8325 strain, and different purification protocols were used for each protein. Highly purified antigens formulated with aluminum hydroxide or adsorbed to SMIP.7–10-Al$(OH)_3$ were used to immunize mice before infection with *S. aureus* strains. 4C-Staph antisera generated in mice or rabbits were used to perform opsonophagocytosis and Hla assays, as well as passive transfer experiments. Splenocytes isolated 10 d after the second immunization of mice were stimulated with Hla_{H35L}, EsxAB, FhuD2, or Csa1A in combination in the presence of Brefeldin A. Experimental methods are described in detail in *SI Materials and Methods*.

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