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Antibodies to *Staphylococcus aureus* capsular polysaccharides 5 and 8 perform similarly *in vitro* but are functionally distinct *in vivo*

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ABSTRACT

The capsular polysaccharide (CP) produced by *Staphylococcus aureus* is a virulence factor that allows the organism to evade uptake and killing by host neutrophils. Polyclonal antibodies to the serotype 5 (CP5) and type 8 (CP8) capsular polysaccharides are opsonic and protect mice against experimental bacteremia provoked by encapsulated staphylococci. Thus, passive immunotherapy using CP antibodies has been considered for the prevention or treatment of invasive antibiotic-resistant *S. aureus* infections. In this report, we generated monoclonal antibodies (mAbs) against *S. aureus* CP5 or CP8. Backbone specific mAbs reacted with native and O-deacetylated CPs, whereas O-acetyl specific mAbs reacted only with native CPs. Reference strains of *S. aureus* and a selection of clinical isolates reacted by colony immunoblot with the CP5 and CP8 mAbs in a serotype-specific manner. The mAbs mediated *in vitro* CP type-specific opsonophagocytic killing of *S. aureus* strains, and mice passively immunized with CP5 mAbs were protected against *S. aureus* bacteremia. Neither CP8-specific mAbs or polyclonal antibodies protected mice against bacteremia provoked by serotype 8 *S. aureus* clinical isolates, although these same antibodies did protect against a serotype 5 *S. aureus* strain genetically engineered to produce CP8. We detected soluble CP8 in culture supernatants of serotype 8 clinical isolates and in the plasma of infected animals. Serotype 5 *S. aureus* released significantly less soluble CP5 *in vitro* and *in vivo*. The release of soluble CP8 by *S. aureus* may contribute to the inability of CP8 vaccines or antibodies to protect against serotype 8 staphylococcal infections.

Introduction

Staphylococcus aureus is a Gram-positive bacterial species that causes multiple infections in humans, ranging from relatively mild infections, such as skin and soft tissue infections, to severe life threatening invasive diseases, such as bacteremia, pneumonia, and endocarditis. Antibiotic therapy to control these infections is currently limited by the widespread emergence of antibiotic resistant *S. aureus* strains. Whereas immunization to prevent staphylococcal infection would be ideal, multiple efforts to produce an effective vaccine have failed to achieve successful endpoints in clinical trials.[1] Current efforts in the vaccine field are focused on multicomponent vaccines that include antigens that provoke opsonic antibodies, neutralize staphylococcal toxins, block bacterial adherence, and elicit an IL-17 response in appropriate T cell populations.[2-4] Immunotherapy represents another

means of addressing the diminishing antibiotic pipeline, and it has the advantage of potential effectiveness in target populations that are incapable of generating a protective immune response due to chronic conditions or various degrees of immune compromise. Monoclonal antibody (mAb) based products have shown efficacy for therapies against cancer, autoimmune and inflammatory disorders, and more recently, viral diseases and bacterial toxins have been successfully targeted.[5-8,9]

There is a clear need for improved immunotherapies against staphylococcal infections, especially those caused by methicillin-resistant *S. aureus* (MRSA) strains. The results of early studies have revealed that targeting a single antigen is not likely to be effective.[10-12] Two separate phase 3 clinical trials attempted to prevent sepsis in low-birth-weight premature neonates by passive immunotherapy targeting surface antigens. INH-A21 is a pooled

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human immunoglobulin preparation enriched for antibodies to the *S. aureus* cell wall anchored clumping factor A protein; Pagibaximab is a humanized mAb that targeted lipoteichoic acid common to several Gram-positive pathogens.[11] Neither product significantly reduced the incidence of staphylococcal sepsis in neonates.

A phase 2 study of tefibazumab, a humanized mAb that binds to clumping factor A, enrolled hospitalized patients with documented *S. aureus* bacteremia.[13] Subjects were randomized to receive either a single dose of tefibazumab plus standard therapy or standard therapy alone. At the conclusion of the trial, composite clinical endpoints between the patients in the tefibazumab group and the placebo group were not significantly different.

Serotype 5 (CP5) or serotype 8 (CP8) capsular polysaccharides are produced by 75–80% of *S. aureus* clinical isolates,[14,15] and capsules have served as effective vaccine targets against other encapsulated bacterial pathogens.[16] Staphylococcal CPs elicit opsonic antibodies,[17] and opsonophagocytic uptake and killing by neutrophils is a key component for host clearance of *S. aureus*. AltaStaph is a hyperimmune polyclonal antibody preparation with high levels of vaccine-induced antibodies to *S. aureus* CP5 and CP8. In a phase 2 study, low-birth-weight neonates were given two intravenous (IV) doses of AltaStaph or placebo.[18] The rates of adverse events between the two arms of the study were similar, and the rates of *S. aureus* bacteremia were nearly identical (∼3%) in both groups. Another phase 2 trial enrolled patients with documented *S. aureus* bacteremia who received standard therapy plus Altastaph or placebo,[19] but the vaccine-induced CP antibodies were insufficient to significantly reduce *S. aureus* bacteremia in this at-risk population.

Human mAbs that neutralize the cytotoxic effects *S. aureus* α hemolysin (Hla) have entered phase 2 clinical trials for the prevention or treatment of staphylococcal pneumonia. Hla mAbs reduced the severity and tissue damage associated with staphylococcal skin infections and necrotizing pneumonia in preclinical studies.[20-22] A human mAb that neutralizes both Hla and four staphylococcal leukocidins[23] is beginning a phase 2 clinical trial for the prevention of *S. aureus* pneumonia in mechanically ventilated subjects. Because of the complexity of *S. aureus* pathogenesis and the diversity of staphylococcal infections, it is likely that immunoprophylaxis with mAbs will need to target multiple staphylococcal antigens.

S. aureus clearance by the host is dependent upon opsonic antibodies that promote uptake and killing of this microbe by host neutrophils. Antibodies to *S. aureus* CP5 and CP8 are particularly effective in promoting opsonophagocytic killing of *S. aureus*[24-26] and reducing bacteremia in a murine infection model.,[25,26] In this report we evaluate mAbs against *S. aureus* CP5 and CP8 for their specificity in binding to a wide variety of encapsulated clinical isolates and their ability to mediate *in vitro* opsonophagocytic bacterial killing. We assessed protection against bacteremia provoked by both serotype 5 and 8 *S. aureus*, and we discovered that serotype 8 *S. aureus* clinical isolates release soluble CP8 during culture and infection. Soluble CP8 released by *S. aureus in vivo* may interfere with protection by CP8 antibodies.

Results

Immunization of Balb/c mice with CP5 or CP8 conjugates

Balb/c mice immunized with CP5 or CP8 conjugate vaccines developed high levels of CP5 or CP8 serum antibodies, respectively (Fig. S1A and S1B). Mice given CP5 conjugated to cross-reactive material 197 (CRM197), a genetically detoxified form of diphtheria toxin, showed a serotype-specific antibody response to CP5. As we reported previously,[26] serum antibodies from mice immunized with CP8-CRM197 showed not only good binding to purified CP8, but also showed some cross-reactive binding to microtiter plates coated with CP5 (Fig. S1A). After hybridoma fusions were performed, two positive clones were selected for their reactivity with CP5, and three were selected for reactivity with CP8. Each of the mAbs was isotype IgG1κ (Table 1).

Bacterial polysaccharides are commonly O-acetylated, and the O-acetyl motif is frequently an immunodominant epitope.[27-30] To determine the specificity of the mAbs, we tested their binding by ELISA to purified native or chemically O-deacetylated CP5 or CP8. Unlike polyclonal sera,[26] none of the mAbs tested showed cross-reactivity between CP5 and CP8 (Fig. 1). mAb CP5–4C2 bound to both native CP5 and O-deacetylated CP5 (Fig. 1B and 1C). However, the binding to the O-deacetylated CP5 was only about half that of the native

Table 1. Monoclonal antibodies isolated from mice immunized with CP5 or CP8 conjugate vaccines.

Monoclonal antibody	Antigen specificity	Subclass	Light chain	Epitope
4C2	CP5	IgG1	kappa	Backbone
5D1	CP5	IgG1	kappa	O-acetyl
5A6	CP8	IgG1	kappa	Backbone
3B5	CP8	IgG1	kappa	Backbone
4G5	CP8	IgG1	kappa	O-acetyl

Figure 1. Reactivity of five mAbs with native and O-deacetylated CP5 and CP8. (A) Structural similarities between CP5 and CP8 and their sites of O-acetylation (O-Ac). Both CP5-specific mAbs (4C2 and 5D1) reacted with (B) native CP5, whereas only mAb 4C2 reacted with (C) O-deacetylated CP5 (De-O-CP5). Three CP8-specific mAbs (5A6, 3B5, and 4G5) reacted with (D) native CP8, whereas only 5A6 and 3B5 reacted with (E) O-deacetylated (De-O-CP8) CP8. D-ManNAcA, N-acetyl-D-mannosaminuronic acid; L-FucNAc, N-acetyl-L-fucosamine; D-FucNAc, N-acetyl-D-fucosamine. Each sample was tested in duplicate, and the ELISAs were performed at least twice. The mean values of a representative experiment are shown.

antigen, suggesting that the epitope recognized by CP5–4C2 may be influenced by the presence of the O-acetyl group on the N-acetyl L-fucosamine residue. In contrast, mAb CP5–5D1 bound to native CP5 but not O-deacetylated CP5. These results indicate that mAb CP5–4C2 was "backbone specific," whereas CP5–5D1 was CP5 O-acetyl specific. Likewise, CP8 mAbs 5A6 and 3B5 reacted with both native and O-deacetylated CP8, and as such were backbone specific (Fig. 1D and 1E).

mAb CP8–4G5 showed binding specificity for native CP8, and it did not react with O-deacetylated CP8. Characteristics of the five mAbs are summarized in Table 1.

Reactivity of mAbs with serotype 5 and 8 S. aureus isolates

Because *S. aureus* isolates have been reported to vary in their degree of CP O-acetylation,[30] we tested the

Table 2. Strains used in this study or tested in the colony immunoblot.

Blot Position	Species, strain	Capsule type	Description or source	Ref. or source
	Staphylococcus spp.			
A3	S. epidermidis RP62A	NA	Biofilm producing, methicillin-resistant strain	[65]
A4	S. haemolyticus JCSC 1435	NA	Sequenced strain from a Japanese patient in Tokyo	[66]
A5	S. saprophyticus 15305	NA	ATCC; genome sequenced uropathogenic isolate	[67]
A6	S. lugdunensis 45–6447	NA	Bacteremia isolate from 2014	BWH[a]
	Staphylococcus aureus			
B1	Newman	5	Human isolate from 1952	[68]
B2	Mu50	5	Vancomycin-intermediate MRSA isolate; Japan	[69]
B3	COL	5	MRSA strain with SCCmecII	[65]
B4	502A	5	Strain adept at colonization	[70]
B5	NRS 382	5	USA100 MRSA	NARSA[b]
B6	NRS 383	5	USA200 MRSA	NARSA
B7	NRS 386	5	USA700 MRSA	NARSA
C1	Lowenstein	5	Nabi serotype 5 reference strain	[71]
C2	Reynolds (CP5)	5	Prototype CP5+ strain	[17]
C3	Reynolds Δcap5H	5	Produces WT levels of O-deacetylated CP5	[51]
C4	Crimson 5313	5	Bacteremia isolate from 2012	BWH
C5	Crimson 18207	5	Bacteremia MRSA isolate from 2013	BWH
C6	29213	5	Antibiotic susceptibility testing reference strain	ATCC
C7	ST22 #15	5	CA-MRSA[c] from 2010; Regensburg, Germany	F. Hanses
D1	MN8	8	Menstrual toxic shock isolate	[72]
D2	ST80–16	8	CA-MRSA strain from 2010; Regensburg, FRG	[73]
D3	Wright	8	Nabi serotype 8 reference strain	[71]
D4	PS80	8	ATCC 27700	[58]
D5	Becker	8	Prototype CP8+ strain	[74]
D6	V8	8	Source of staphylococcal V8 protease	[75]
D7	HS-522	8	ST239 MRSA	[76]
E1	NRS 387	8	USA800 MRSA	NARSA
E2	NRS 483	8	USA1000 MRSA	NARSA
E3	NRS 484	8	USA1100 MRSA	NARSA
E4	GFRY	8	Bacteremia isolate from 1997	BWH
E5	VP	8	Bacteremia isolate from 1995	[41]
E6	JP122	8	Bacteremia isolate from 1990	BWH
E7	JP136	8	Bacteremia isolate from 1990	BWH
F1	25923	8	Seattle 1945; antibiotic reference strain	ATCC
F2	Reynolds (CP8)	8	Reynolds genetically modified to produce CP8	[17]
F3	UAMS-1	8	Genome sequenced musculoskeletal isolate	[77]
F4	2672	8	USA400; necrotizing pneumonia and sepsis isolate	[32]
F5	RF122	8	Genome sequenced bovine isolate	[78]
F6	Sanger 252	8	Epidemic MRSA clone	[31]
F7	MW2	8	Community acquired MRSA; USA400	[79]
G1	Sanger 476	CP-	Community acquired MSSA; USA400	[31]
G2	8325–4	CP-	NCTC; cured of three prophages	[80]
G3	LAC	CP-	Los Angeles clone; USA300 MRSA	[81]
G4	NRS 482	CP-	USA300 MRSA	NARSA
G5	NRS 691	CP-	USA500 MRSA	NARSA
G6	Cowan I	CP-	Isolate with high protein A expression	[82]
G7	Reynolds (CP-)	CP-	Reynolds acapsular mutant	[17]

*Note.* [a]BWH, Brigham and Women's Hospital, Boston, MA.
[b]NARSA, Network on Antimicrobial Resistance in *Staphylococcus aureus*.
[c]CA-MRSA, community-acquired methicillin resistant *Staphylococcus aureus*.

reactivity of the backbone specific mAbs CP5–4C2 and CP8–5A6 against a range of *S. aureus* isolates (Table 2) by a colony immunoblot method. As shown in Fig. 2, neither mAb reacted with representative strains of coagulase-negative staphylococci, including *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus saprophyticus*, or *Staphylococcus lugdunensis*. mAb CP5–4C2 reacted with all 14 serotype 5 *S. aureus* strains tested, including reference strains (such as Newman and Lowenstein), clinical isolates, and MRSA. Likewise, mAb CP8–5A6 reacted with 19 of 21 serotype 8 strains, including MRSA strains. Serotype 8 Sanger 252 (position F6 on the immunoblot), among the most genetically diverse sequenced *S. aureus* strains,[31] reacted weakly with mAb CP8–5A6. Sanger 252 produces scant CP8, as discussed below and depicted in Fig. 7. *S. aureus* strain MW2 (position F7 on the immunoblot) also reacted poorly with mAb CP8–5A6, consistent with its minimal reactivity with CP8 polyclonal antibodies.[32] MW2 carries a frame-shift mutation in cap8A, resulting in a truncated version of the cap8A gene product. Strain MW2 produces scant CP8 that is reduced in molecular size compared with that of other serotype 8 isolates (J. Lee, unpublished observations).
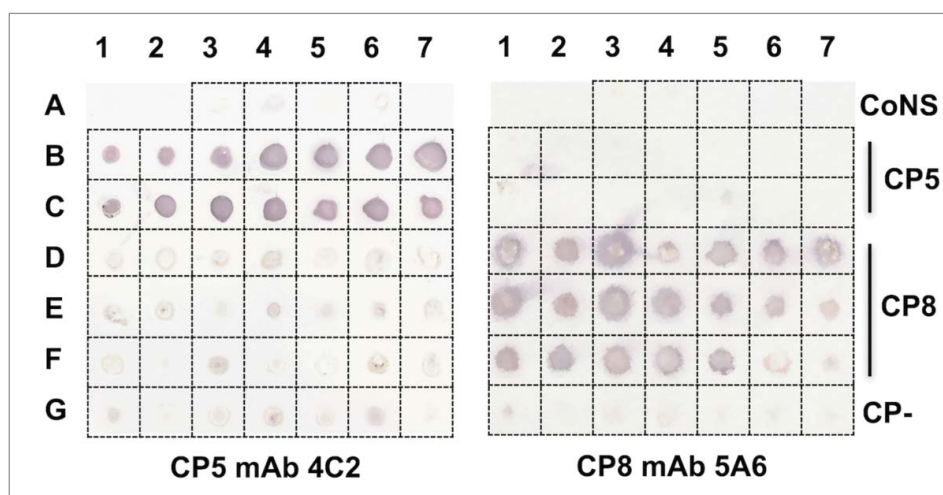
Figure 2. mAbs CP5–4C2 and CP8–5A6 were tested by colony immunoblot for reactivity with *S. aureus* type 5 and 8 reference strains or clinical isolates. The staphylococcal isolates are listed in Table 2. mAb 4C2 reacted with all 14 serotype 5 isolates, whereas mAb 5A6 reacted with 19 of 21 serotype 8 isolates. CoNS, coagulase-negative staphylococci; CP-, capsule negative. The immunoblot was performed three times, and a representative blot is shown.

Opsonophagocytic killing (OPK) of S. aureus mediated by mAbs

Previous studies have demonstrated that polyclonal antibodies to staphylococcal CPs are opsonic for encapsulated *S. aureus*.[24-26] To determine whether the CP5- and CP8-specific mAbs were functionally opsonic, we performed *in vitro* OPK assays with agar-grown *S. aureus* and serial dilutions of either a purified backbone-specific or O-acetyl-specific mAb. Opsonic activity was compared by determining the mAb concentration that yielded 50% killing of the bacterial inoculum. In the presence of HL60 cells and a complement source (guinea pig serum), backbone-specific mAb CP5–4C2 showed equivalent opsonic killing activity against strains *S. aureus* Reynolds (CP5) and the O-deacetylated Δcap5H

mutant (Fig. 3A). O-acetyl-specific mAb CP5–5D1 showed good opsonic activity against Reynolds (CP5), but it mediated little opsonic killing against the Δcap5H mutant, with loss of activity at concentrations <250 ng/ml (Fig. 3B). These data are consistent with our ELISA results that mAb CP5–5D1 bound only to native O-acetylated CP5.

Because the backbone-specific mAb CP5–4C2 has the advantage of opsonic activity against serotype 5 *S. aureus* strains independent of CP5 O-acetylation, mAb 4C2 was tested further in OPK assays comparing the heavily encapsulated strain Reynolds (CP5) with strain Newman, which produces little CP5 (see Fig. 7). Whereas strain Reynolds (CP5) was opsonized for OPK by CP5–4C2 concentrations as low as 40 ng/ml, strain
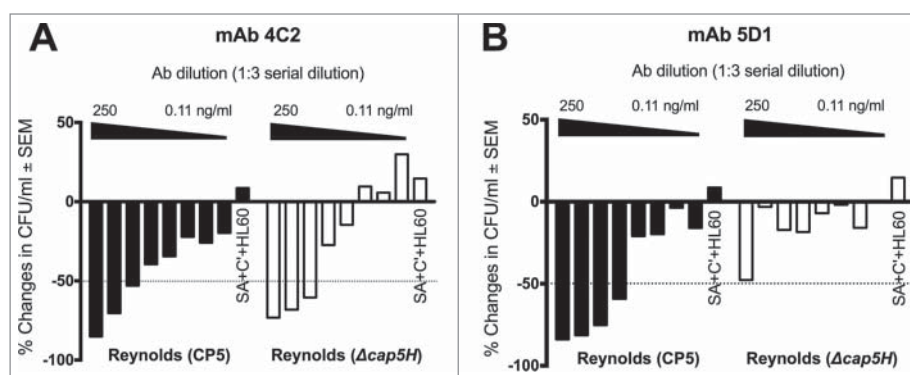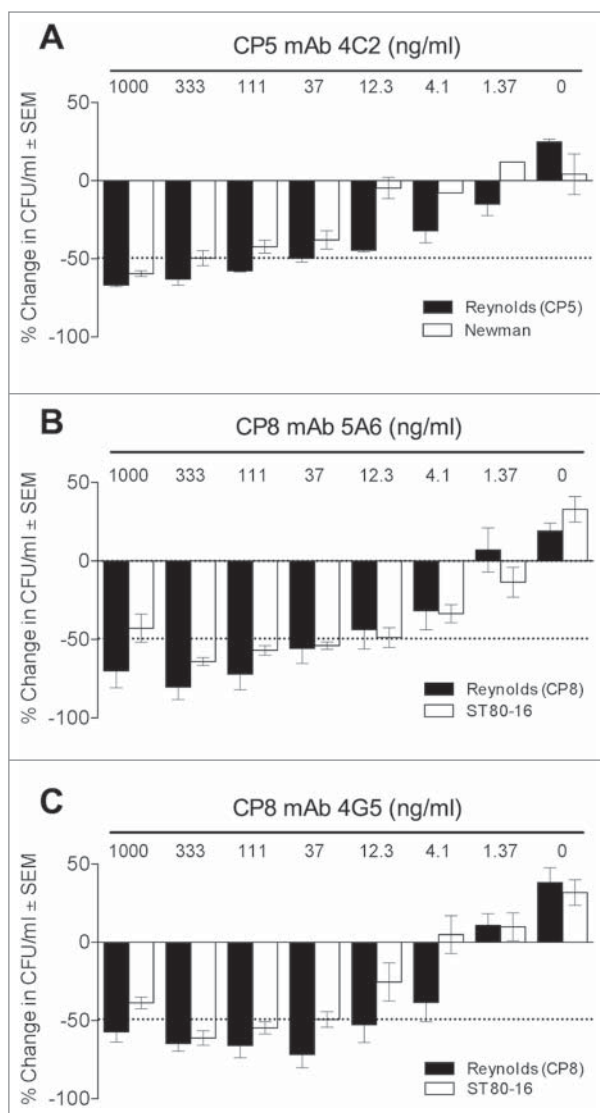

Figure 3. Opsonic activity of CP5-specific mAbs (A) 4C2 and (B) 5D1 against wild type strain Reynolds (CP5) and its isogenic *cap5H* deletion mutant that produces wild-type levels of O-deacetylated CP5. The mAbs were serially diluted 3-fold and tested in the OPK assay. The results are expressed as percent changes in the number of CFU/ml after a 2-h incubation with mAb, HL60 cells, and a complement (C′) source. The samples labeled SA+ C′+HL60 contain no mAb. The titer is expressed as the lowest mAb dilution that killed 50% of the inoculum (dotted line). The experiment was performed twice, and a representative experiment is shown.

Newman was killed in the assay by mAb CP5–4C2 at concentrations ≥ 333 ng/ml (Fig. 4A). Because the target antigen is not expressed abundantly in strain Newman, this may explain its requirement for a higher CP5 mAb concentration to achieve OPK activity. mAb CP5–4C2 (1 μg/ml) was not opsonic for Reynolds (CP-) or USA300 strain LAC (data not shown), since both these strains lack a capsule. There was little to no bacterial killing of the encapsulated *S. aureus* strains in the presence of complement (C'), bacteria, and phagocytes, but no


Figure 4. Comparative opsonic activity of mAbs against serotype 5 and 8 *S. aureus* strains. (A) Backbone-specific CP5 mAb 4C2 showed somewhat better opsonic activity against Reynolds (CP5) than strain Newman. (B) Backbone-specific CP8 mAbs 5A6 showed comparable opsonic activity against Reynolds (CP8) and MRSA strain ST80–16. (C) O-acetyl-specific CP8 mAb 4G5 showed greater OPK activity against Reynolds (CP8) than strain ST80–16. The sample labeled 0 antibody contained HL60s, *S. aureus*, and the complement source. The data shown are means (± standard errors) of three to four experiments.

antibody (Fig. 4) or in the presence of antibody alone. Thus the bacterial killing measured in the OPK was not the result of antibody-mediated bacterial agglutination, complement mediated opsonic activity, or mAb bactericidal activity.

CP8-specific mAb CP8–5A6 is backbone specific (Fig. 1D and Fig 1E); in OPK assays it was opsonic for both Reynolds (CP8) and MRSA strain ST80–16 at mAb concentrations as low as 12 ng/ml (Fig. 4B). Similarly, the O-acetyl specific mAb CP8–4G5 was also opsonic for Reynolds (CP8) at concentrations as low as 12 ng/ml (Fig. 4C). However, the opsonic activity of mAb CP8–4G5 against *S. aureus* ST80–16 was somewhat less, showing 50% killing at a concentration of 37 ng/ml, possibly reflecting a lower level of CP8 O-acetylation than Reynolds (CP8). Moreover, both CP8 mAbs 5A6 and 4G5 showed diminished opsonic activity at the highest concentration tested (1 μg/ml), although the reduction was only significant with strain ST80–16 and CP8 mAb 4G5 (P < 0.05 by one-way ANOVA). The CP- strain LAC was not opsonized for killing by CP8-specific mAbs 5A6 or 4G5 (data not shown).

To determine whether the mAbs would opsonize *S. aureus* that were not cultivated under conditions that maximize CP production, we performed additional OPK assays with staphylococcal strains cultivated overnight in tryptic soy broth (TSB). As shown in Fig. S2A, broth-grown *S. aureus* Reynolds (CP5) and Newman were still opsonized for phagocytic killing by mAbs CP5–4C2, although bacterial killing was reduced when compared with bacteria grown on CSA plates (Fig. 4), a growth condition optimal for CP production. Similarly, CP8 mAb 5A6 (Fig. S2B) showed somewhat reduced opsonic activity against broth-grown *S. aureus* Reynolds (CP8) compared with agar-grown bacteria. Of note, when MRSA strain ST80–16 was grown in TSB for the OPK, the addition of CP8-specific mAb 5A6 to the sample did not enhance the killing obtained in the absence of antibody (0 mAb), suggesting that little or no surface associated CP8 is present on the bacterium under these culture conditions. *S. aureus* ST80–16 and other isolates were further characterized for CP production in broth culture in experiments detailed below.

The effect of CP-specific antibodies in a mouse model of *S. aureus* bacteremia

We reported previously that polyclonal antibodies to CP5 significantly reduced bacteremia in mice challenged with Reynolds (CP5), Newman, USA100, or USA200.[25,26] Therefore, the CP5 backbone specific mAb 4C2 was evaluated for its ability to protect mice against bacteremia in passive immunization experiments. As

shown in Fig. 5A, mice injected IV with 100 μg of irrelevant mAb 6C5 developed bacteremia that was measured 2 h after intraperitoneal challenge with Reynolds (CP5). In contrast, mice that received 100 μg CP5 mAb 4C2 or 300 μg of rabbit IgG to CP5 conjugated to Pseudomonas aeruginosa exoprotein A (CP5-Epa) showed significant protection against bacteremia. Likewise, mice given 100 μg of the CP8 backbone-specific mAb 5A6 showed protected against Reynolds (CP8) bacteremia compared with mice given irrelevant mAb 6C5 (Fig. 5B).

Because there is a paucity of evidence supporting the protective efficacy of CP8 antibodies,[33] we examined
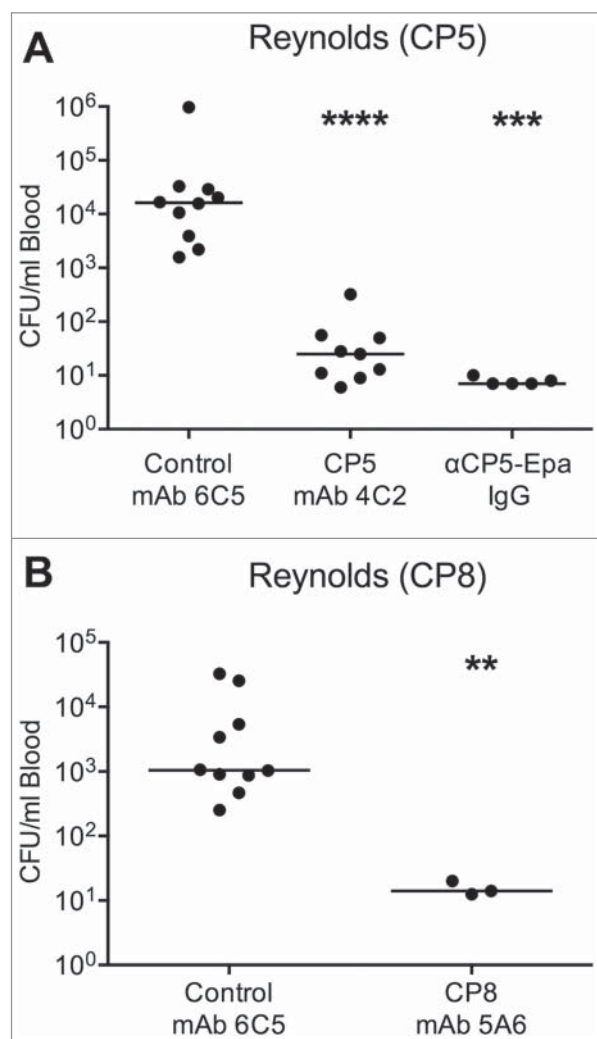
Figure 5. Passive immunization with CP5- or CP8-specific mAbs in the mouse bacteremia model. Mice were immunized IV with 100 μg of (A) CP5 mAb 4C2 or (B) CP8 mAb 5A6 24 h before challenge by the IP route with 10^7 CFU (A) *S. aureus* Reynolds (CP5) or (B) Reynolds (CP8). Control mice were immunized with irrelevant mAb 6C5 or polyclonal CP5 specific rabbit IgG (300 μg), and all mice were bled 2 h after bacterial challenge. The horizontal lines represent median CFU/ml blood for each group of mice. Bacteremia levels in mice administered mAbs 4C2 or 5A6 were compared by the Mann-Whitney U test to levels in control mice given mAb 6C5. ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$.

whether CP8 mAb 5A6 would reduce bacteremia provoked by additional serotype 8 isolates. Compared to mice given isotype control mAbs, mice passively immunized with 100 μg CP8 mAb 5A6 and challenged 24 h later with serotype 8 *S. aureus* strains ST80–16, MN8, or Wright showed no significant differences in bacteremia levels (Fig. S3). To address whether the CP8 mAbs might be less effective *in vivo* than CP8-specific polyclonal antibodies, additional mice were passively immunized IV with 1 mg of either polyclonal rabbit IgG to CP8-Epa or polyclonal IgG raised to an irrelevant antigen (Shigella 2a-Epa). Mice given CP8-Epa antibodies and challenged with Reynolds (CP8) showed a median reduction of 98% (1.72 $\log_{10}$) in CFU/ml blood after 2 h compared with mice given the irrelevant IgG (Fig. 6A). Consistent with our mAb 5A6 results, however, none of the mice challenged with ST80–16 (low inoculum of 7×10^7 CFU/mouse or high inoculum of 1×10^8 CFU/mouse) were protected against bacteremia by 1 mg polyclonal CP8 antibodies (Fig. 6B, C). These results indicate that the serotype 8 clinical *S. aureus* isolates are distinct from the genetically engineered Reynolds (CP8) strain as a target for CP8 antibodies.

Reynolds (CP8) is not typical of clinical CP8-producing *S. aureus* isolates

We constructed Reynolds (CP8) from the parental serotype 5 strain Reynolds by genetically swapping the serotype 5-specific cap5HIJK genes from Reynolds with the serotype 8-specific cap8HIJK genes from strain Becker.[17] We measured cell-associated CP and soluble CP released into supernatants of overnight cultures of Reynolds (CP5), Reynolds (CP8), as well as 14 additional *S. aureus* strains. Whereas our ability to reliably measure soluble CP in complex media like TSB was poor, we could readily measure CP5 or CP8 in the supernatants of *S. aureus* cultivated overnight in RPMI + 1% casamino acids. We report CP levels relative to the isogenic Reynolds (CP5) and Reynolds (CP8) strains (included in every assay) to normalize the results from multiple experiments performed on each strain. As shown in Fig. 7A and 7C, clinical *S. aureus* isolates vary markedly in bacterial cell-associated CP levels, and all of the strains produced less CP than Reynolds (CP5) or Reynolds (CP8). Cell-associated CP5 was ~5-fold higher for the hospital-associated MRSA strains USA100 (NRS 382) and USA200 (NRS 383) strains compared with strains Newman or the MRSA strain ST22. No detectable CP5 was produced by USA300 strain LAC, consistent with previous reports.[32,34] Similar variability was seen among the nine serotype 8 strains tested. All produced less cell-associated CP8 than did Reynolds (CP8); Sanger 252 and two of the three ST80 clinical isolates tested produced only trace amounts of cell-associated CP8.
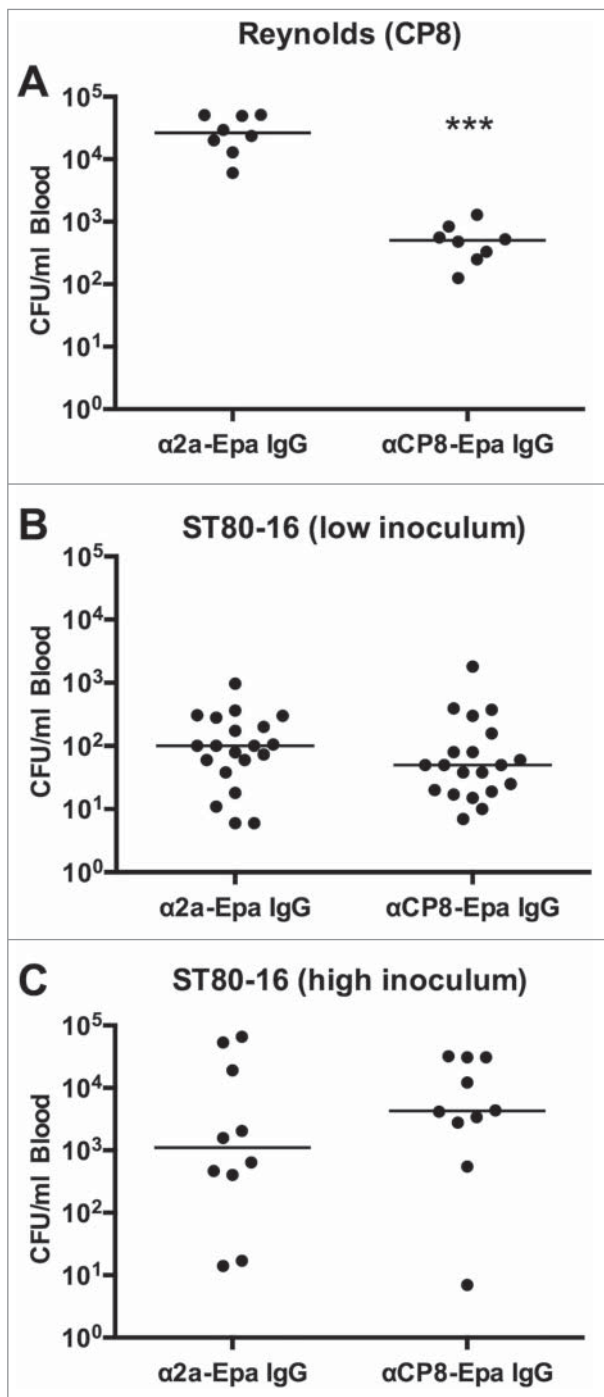
Figure 6. Passive immunization with polyclonal anti-CP8 antibodies in the mouse bacteremia model. Mice were immunized with 1 mg anti-CP8-Epa or anti-Shigella 2a-Epa IgG 24 h before challenge with 10^7 CFU (A) *S. aureus* Reynolds (CP8) or (B, C) two different inocula (low inoculum 7×10^7 CFU/mouse or high inoculum 1×10^8 CFU/mouse) of strain ST80–16. Mice challenged with Reynolds (CP8) were bled 2 h after bacterial challenge, and mice challenged with ST80–16 were bled 1.5 h after bacterial challenge. The horizontal lines represent median CFU/ml blood for each group of mice. Bacteremia levels in mice given CP8-Epa IgG were compared by the Mann-Whitney U test to levels in control mice given 2a-Epa IgG. ***, $P < 0.001$.

When we measured soluble CP5 in overnight culture supernatants of serotype 5 *S. aureus* strains (Fig. 7B), CP5 levels in the supernatant of most of the isolates mirrored levels produced by Reynolds (CP5). Strain Newman released only 31% of the CP5 levels released by Reynolds (CP5), but Newman also made little cell-associated CP5 (Fig. 7A).

Reynolds (CP8) released soluble CP in quantities similar to those produced by the parental strain Reynolds (CP5). In contrast, there was a broad range of soluble CP8 levels detected in the culture supernatants of the serotype 8 clinical isolates (Fig. 7D), and all of the isolates released more CP8 than strain Reynolds (CP8). Three of the strains (Sanger 252, NRS 483, and MN8) released >2-fold more CP8 than Reynolds (CP8), but these differences did not reach statistical significance. The remaining isolates released 4- to 10-fold more CP8 than Reynolds (CP8) under these *in vitro* conditions. We observed no consistent correlations between cell associated and soluble CP produced by the *S. aureus* isolates examined. Nonetheless, for the CP8+ strains (with the exception of Sanger 252), strains with little cell-associated CP8 (Wright, ST80–13, and ST8–17) showed the highest levels of soluble CP8.

CP antigenemia

Arbeit and coworkers reported that CP8 was present in the serum of experimental animals infected with serotype 8 *S. aureus* strains.[35,36] To determine whether CP8 was present in higher concentrations than CP5 in the blood of bacteremic animals, we infected groups of 10–11 mice with NRS 382 (CP5+) or ST80–17 (CP8+). When the mice became moribund (1 to 5 d post-challenge), the animals were killed. Quantitative cultures of the infected mouse blood showed that mice infected with the NRS 382 strain had a mean log CFU/ml (± SEM) of 5.20 ± 0.44 in the blood, whereas mice infected with ST80–17 had a mean log CFU/ml of 5.21 ± 0.32 *S. aureus*. Plasma CP levels in both groups were quantified by ELISA. As shown in Fig. 8, CP8 levels in the plasma of ST80–17 infected animals were significantly (P = 0.0309) higher than CP5 levels in the blood of mice infected with NRS 382. Two of 11 animals had detectable CP5 in the mouse plasma (one mouse had 110 ng CP5 per ml blood), whereas 7 of 10 mice infected with ST80–17 had detectable CP8 levels (P = 0.03; Fisher exact test).

Discussion

CP5 and CP8 are expressed by many *S. aureus* clinical isolates,[14,15,37] and CPs promote bacterial survival in the blood.[17,24] CPs are important in immune evasion,
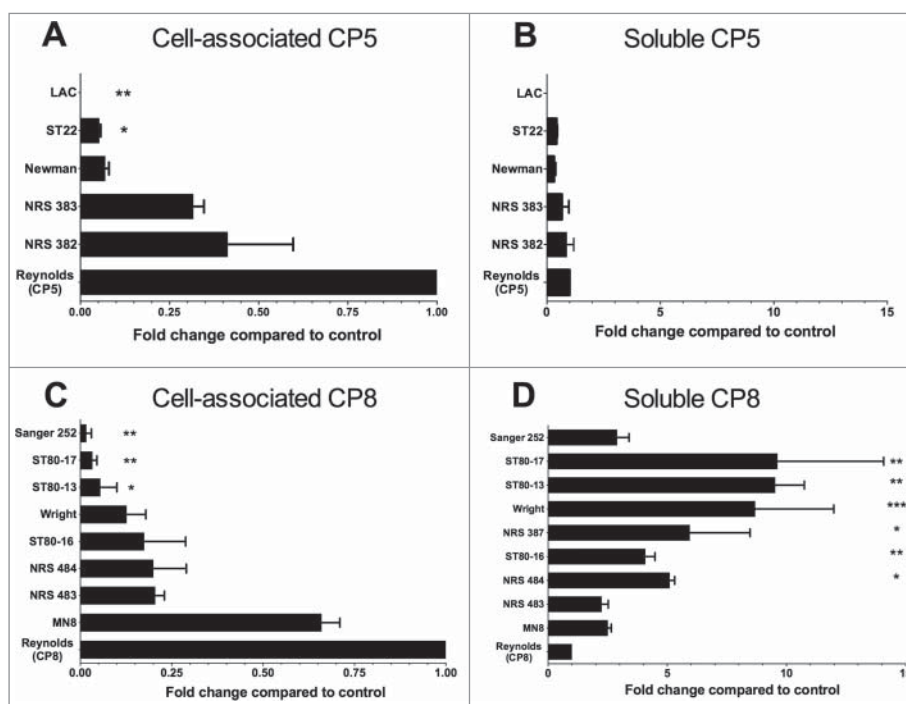
Figure 7. Quantitation of cell-associated and soluble CP5 or CP8 produced by clinical isolates of *S. aureus* cultivated in RMPI + 1% casamino acids. CP levels were measured by an ELISA inhibition method, and CP5 and CP8 concentrations are expressed relative to those of Reynolds (CP5) and Reynolds (CP8), respectively, which were included as reference strains on every assay. CP levels were compared by one-way ANOVA, and multiple comparisons were made to the reference strains Reynolds (CP5) and Reynolds (CP8). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

allowing the bacterium to evade uptake by professional phagocytes.[17,24,25,38] Antibodies to CPs neutralize the antiphagocytic nature of the capsule and effectively mediate uptake and killing by professional phagocytes. Passive immunization with CP5 or CP8 antibodies has shown protection in rodent models of mastitis, bacteremia, endocarditis, and skin abscesses.[25,39-41]
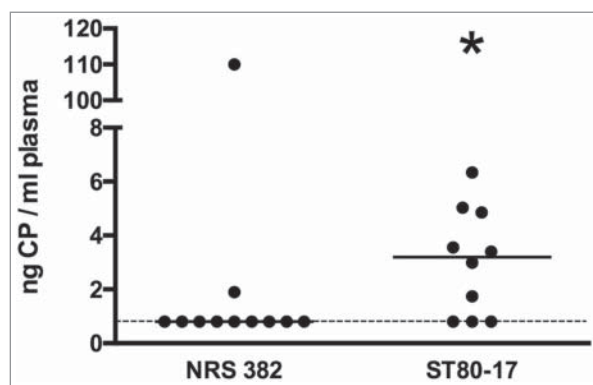
Figure 8. Plasma levels of CP5 or CP8 in mice infected IV with $\sim 2 \times 10^8$ CFU of *S. aureus* NRS382 (CP5+) or ST80–17 (CP8+), respectively. After 1 to 5 days, the animals were bled by cardiac stick, and the plasma extracts were prepared. CP levels were determined by an ELISA inhibition assay, and CP concentrations were compared by the Mann-Whitney U test. *, $P < 0.05$. The lower limit of CP detection is indicated by a dashed line.

Despite their failure in clinical trials when used alone in hemodialysis patients,[42,43] CP5 and CP8 conjugate vaccines are thought to be important components in a multivalent staphylococcal vaccine.[1,25,38,44] Because diverse *S. aureus* clinical isolates (both methicillin-sensitive and –resistant) produce surface-associated CP5 or CP8,[14,15,37] we considered that mAbs to CP5 or CP8 with opsonic activity might be included in a mAb cocktail to prevent or reduce staphylococcal bacteremia. *S. aureus* USA300, which is highly prevalent in the United States, does not produce a capsule and thus would not be a target for mAbs against CP5 or CP8.[34] However, the majority of USA300-associated infections involve superficial wounds or abscesses,[45] and USA300 isolates are not common outside of North America.[46-48] Among predominant MRSA clones worldwide are the CP8+ lineages ST1, ST30, ST59, ST80, and ST239 and the CP5+ lineages ST5 and ST22.

mAbs against other staphylococcal virulence factors, such as protein A, clumping factor A, and leukocidins have been evaluated in preclinical studies for passive immunotherapeutic purposes.[23,49,50] Considering the complexity of the *S. aureus* disease spectrum, it is likely that a cocktail of mAbs targeting distinct staphylococcal virulence factors may serve to protect against diverse

infections, and this cocktail might be delivered therapeutically along with conventional antimicrobial agents.

In the generation of murine mAbs specific for *S. aureus* CP5 or CP8, we noted that some of the mAbs recognized the immunodominant O-acetyl epitope that decorates the backbone structure of CP5 and CP8. O-acetylation of bacterial polysaccharides is common, and the O-acetyl motif is known to be highly immunogenic in encapsulated bacteria.[27-30] Bhasin et al. reported that CP5 O-acetylation rendered *S. aureus* more resistant to opsonophagocytic killing (OPK) *in vitro* and enhanced bacterial virulence in a murine infection model.[51] *S. aureus* CP5 and CP8 have similar trisaccharide repeating units.[52] However, CP5 is O-acetylated on the N-acetyl fucosamine residue, whereas CP8 is O-acetylated on the N-acetyl mannosaminuronic acid (Fig. 1A). Consequently, the O-acetyl-specific CP5 mAb 5D1 and CP8 mAb 4G5 did not cross react between CP5 and CP8. Both O-acetyl and backbone-specific mAbs demonstrated binding and opsonic activity as measured by *in vitro* assays. However, both CP8 mAbs 5A6 and 4G5 showed diminished *in vitro* opsonic activity at the highest concentration tested (1 μg/ml), although the reduction was only significant with strain ST80–16 and O-acetyl specific CP8 mAb 4G5. Although this prozone phenomenon has been reported previously,[53,54] its mechanism is poorly understood. Some reports have indicated that excess antibody interferes with C3b deposition.[55,56] We chose to focus on the backbone-specific CP5 and CP8 mAbs for *in vivo* protection assays, since *S. aureus* isolates have been reported to vary in their degree of CP O-acetylation.[30]

We chose the bacteremia model for our *in vivo* studies because *S. aureus* CPs have been shown to markedly enhance bacterial virulence in this model.[17,24] Polyclonal antibodies to the staphylococcal CPs protect mice against bacteremia but not lethal pneumonia,[25] whereas antibodies to *S. aureus* Hla protect mice against lethal pneumonia but not bacteremia.[21,22,25] We showed previously that polyclonal CP5-specific antibodies protected against experimental bacteremia induced by four different serotype 5 isolates.[25] In this study, the CP5 backbone-specific 4C2 mAb protected mice against experimental Reynolds (CP5) bacteremia. Although CP5 mAb 4C2 reacted consistently with 14 serotype 5 *S. aureus* isolates by colony immunoblot, the protective efficacy of the CP5 mAb 4C2 against bacteremia may be impacted by the fact that its binding to the O-deacetylated CP5 was only ~50% of its binding to native CP5 by ELISA.

The CP8-specific mAb 5A6 protected against bacteremia when mice were challenged with Reynolds (CP8), a strain constructed from the parental Reynolds (CP5) strain by genetically swapping the serotype 5-specific cap5HIJK genes from Reynolds with the serotype 8-specific cap8HIJK genes from strain Becker.[17] The genetic backgrounds of these two strains are identical, and so Reynolds (CP5) and Reynolds (CP8) produce similar levels of cell-associated CP,[17] allowing us to evaluate the virulence and biologic activities associated with capsule production in these isogenic strains.[17] However, when mice passively immunized with CP8-specific mAb 5A6 were challenged with three other *S. aureus* isolates (MN8, ST80–16, or Wright), no protection against bacteremia was observed. This finding is consistent with a report by Cook et al.[33] in which 800 μg of a CP8 mAb or goat anti-CP8 IgG delivered by the intraperitoneal (IP) route did not protect mice against a lethal intravenous dose of *S. aureus* MCL8538 (a CP8+ MLST15 skin isolate). Similarly, our data showing that polyclonal CP8 antiserum significantly reduced Reynolds (CP8) bacteremia but not bacteremia provoked by strain ST80–16 suggest that the lack of protective efficacy against clinical *S. aureus* isolates is not limited to CP8 mAbs.

To address the inconsistencies in the protective efficacy of antibodies to CP5 and CP8, we measured both cell-associated and cell-free (soluble) CP produced by a subset of serotype 5 and 8 *S. aureus* strains cultivated in broth culture. Predictably, cell-associated CP levels varied markedly among the different staphylococcal strains. Little soluble CP5 was released by the serotype 5 isolates, and there was little variation among the different strains. In contrast, the serotype 8 strains were characterized by the release of 2- to 10-fold more soluble CP than the control strain Reynolds (CP8). To determine whether this finding was relevant to *in vivo* conditions, we measured CP levels in the plasma of infected mice. Consistent with our *in vitro* findings, CP8 was detected in the plasma of bacteremic mice at higher levels and more consistently than CP5.

We hypothesize that soluble CP8 could bind opsonic CP8-specific antibodies *in vivo* and thus compete and interfere with antibody binding to the encapsulated bacterial surface. Only antibodies bound to the bacterial surface are opsonic, mediating uptake and killing by professional phagocytes. As such, released CP8 would compete and interfere with the opsonic function of capsular antibodies. This scenario would reduce the *in vivo* efficacy of passively administered capsular antibodies, and this premise is consistent with our data demonstrating the inability of CP8 antibodies (polyclonal or mAbs) to protect against experimental bacteremia provoked by clinical CP8+ *S. aureus* strains. The release of CP by a serotype (ST) 3 strain of Streptococcus pneumoniae, but not by ST1, ST4, ST6B, or ST14 strains, was recently reported by Choi et al.[57] Clearly, further work is needed to attain a better understanding of the protective efficacy of CP-specific antibodies, as well as the biosynthetic steps by which CP polymers are anchored (or not) to the cell wall.

Materials and methods

Bacterial strains and growth conditions

The staphylococcal strains used in this study are listed in Table 2. The isolates were grown at 37°C on tryptic soy agar for colony immunoblot testing or on Columbia agar + 2% NaCl plates for OPK assays and for challenge in the mouse bacteremia model. To evaluate the influence of culture conditions on OPK results, some of the assays were repeated with S. aureus cultivated overnight with aeration in TSB. For the measurement of S. aureus cell-associated vs. soluble CP, the strains were cultivated for 24 h with aeration in RPMI1640 medium (Mediatech) supplemented with 1% casamino acids (Becton, Dickinson and Co.).

mAbs

Female Balb/c mice (6 to 8 weeks old; Taconic Farms) were immunized every two weeks by the IP route with 2 μg CP5 conjugated to cross-reactive material 197 (CRM197) or CP8-CRM197 (Wyeth-Pfizer; Collegeville, PA) adsorbed to Alhydrogel, as described.[26] After the fourth immunization, mouse sera were collected, serially diluted, and tested for binding to CP5 or CP8 by ELISA. Three weeks after the fourth immunization, the mice were administered a final booster dose without adjuvant.

Four days after the final immunization, the splenocytes were fused with a myeloma cell line and then subcloned by standard methods. Hybridoma cells were cultured at 37°C in 5% CO2 in RPMI 1640 and supplemented with 10% heat inactivated fetal bovine serum (Hyclone), L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Mediatech). Hybridoma culture supernatants were screened by ELISA for reactivity with CP5 or CP8 that were purified and chemically O-deacetylated as described previously.[58] ELISAs were performed on 96-well plates coated with purified CPs (4 μg/ml) coupled to poly-L-lysine as described.[59] Antigen-specific clones were subcloned by limiting dilution to yield mAb-secreting hybridomas from single cells. Antibodies from the culture supernatants were purified over protein G columns (GE Healthcare). Protein concentrations were assessed by nanodrop (Thermo Scientific), and mAb purity was confirmed by SDS-PAGE (Fig. S4). CP5 and CP8 ELISAs were used to determine mAb concentrations based on an IgG1 standard curve.[60] The colony immunoblot assay [61] and the HL60-based OPK assay [25] were performed as we described previously.

Quantification of S. aureus CP5 or CP8

To quantify cell-associated CP5 or CP8, ELISA inhibition assays were performed on bacterial cells harvested from 24-h cultures, as described.[62,63] To quantify soluble CP, S. aureus culture supernatants were filter-sterilized and then boiled for 10 min to denature proteins and inactivate proteases. Sterile culture medium was used as a negative control. Three-fold serial dilutions of the supernatants or purified CP were then incubated with polyclonal rabbit antibodies specific for CP5 or CP8. ELISA inhibition assays that included a purified CP standard curve were performed as described.[63] CP concentrations were calculated from the linear range of the CP standard curve, and cell-associated and soluble CP concentrations were expressed relative to the control strains Reynolds (CP5) and Reynolds (CP8), included in each assay.

Murine bacteremia model

Animal experiments were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and the protocols were approved by the Harvard Medical School Standing Committee on Animals. Female Swiss Webster mice (7 to 8 week old; Charles River Laboratories) were injected intravenously with 100 μg mAbs 4C2 (CP5-specific) or 5A6 (CP8-specific) or an irrelevant IgG1 mAb control.[64] For certain experiments, mice were injected similarly with 0.3 to 1 mg polyclonal CP5-Epa, CP8-Epa, or Shigella 2a-Epa rabbit IgG, purified by a Protein A affinity chromatography, as described.[25] After 24 h the mice were challenged IP with a 500 μl S. aureus inoculum. Heparinized mouse blood was obtained by tail vein puncture at either 1, 1.5. or 2 h (depending on the S. aureus challenge strain) after bacterial inoculation and cultured quantitatively.

For the detection of CP antigenemia, mice were challenged intravenously (IV) with ~1.6 × 10^8 CFU of S. aureus NRS 382 (CP5+) or ST80–17 (CP8+). Between 1 and 5 d post-inoculation, the mice were killed by CO$_2$ asphyxiation, and heparinized blood was collected from individual mice by cardiac puncture. After quantitative cultures of the blood were performed, the plasma was separated by centrifugation and stored at −20°C. CP antigenemia was detected by the method described by Arbeit and Nelles.[36] Briefly, the plasma was diluted in buffer and then treated with Pronase (Sigma) before autoclaving for 3 min. The samples were clarified by centrifugation and assayed by a capture ELISA. The CP concentrations were determined by comparison with a standard curve of purified CP5 or CP8 added to normal mouse plasma and extracted in a similar fashion.

Statistical analyses

ELISA and OPK data were analyzed using the unpaired two-tailed Student t-test. Quantitative cultures of mouse

blood and concentrations of CP in plasma were compared by the Mann-Whitney U test; CP antigenemia was compared by the Fisher's exact test. Cell-associated and supernatant levels of CPs were analyzed by one-way ANOVA. Multiple comparisons were made to CP levels produced by the reference strains Reynolds (CP5) and Reynolds (CP8). The data were analyzed with Prism 6 (GraphPad Software, Inc.), and P values < 0.05 were considered significant.

Abbreviations

CP	capsular polysaccharide
CRM197	cross reacting material 197
ELISA	enzyme-linked immunosorbent assay
Epa	Pseudomonas aeruginosa exoprotein A
IP	intraperitoneal
IV	intravenous
mAb	monoclonal antibody
MRSA	methicillin-resistant *S. aureus*
NARSA	Network on Antimicrobial Resistance in *S. aureus*.
OPK	opsonophagocytic killing

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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