Staphylococcus aureus vaccines: Deviating from the carol

Dominique Missiakas and Olaf Schneewind

Department of Microbiology, University of Chicago, Chicago, IL 60637

***Staphylococcus aureus*, a commensal of the human nasopharynx and skin, also causes invasive disease, most frequently skin and soft tissue infections. Invasive disease caused by drug-resistant strains, designated MRSA (methicillin-resistant *S. aureus*), is associated with failure of antibiotic therapy and elevated mortality. Here we review polysaccharide–conjugate and subunit vaccines that were designed to prevent *S. aureus* infection in patients at risk of bacteremia or surgical wound infection but failed to reach their clinical endpoints. We also discuss vaccines with ongoing trials for combinations of polysaccharide–conjugates and subunits. *S. aureus* colonization and invasive disease are not associated with the development of protective immune responses, which is attributable to a large spectrum of immune evasion factors. Two evasive strategies, assembly of protective fibrin shields via coagulases and protein A–mediated B cell superantigen activity, are discussed as possible vaccine targets. Although correlates for protective immunity are not yet known, opsonophagocytic killing of staphylococci by phagocytic cells offers opportunities to establish such criteria.**

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Commensalism and disease

Staphylococcus aureus is first and foremost a commensal, colonizing the anterior nares, throat, skin folds, and gastrointestinal tract of humans (Crossley and Solliday, 1980; Wertheim et al., 2005; Peters et al., 2013). Approximately one third of the population is colonized (Gorwitz et al., 2008). Serum analysis of healthy infant or adult carriers and noncarriers revealed that colonization stimulates IgG1 and IgG4 antibody responses against several secreted staphylococcal antigens (Verkaik et al., 2010; Swierstra et al., 2015). Nonetheless, development of such antibody responses is not thought to impact colonization (Swierstra et al., 2015). Colonization increases the risk for *S. aureus* disease, most frequently manifest as skin and soft tissue infection (SSTI; Wertheim et al., 2005). A key feature of *S. aureus* infection is its recurrence, even in patients with effective surgical and antibiotic therapy (Fowler et al., 1999). Annual incidence of SSTI in the US Veterans Administration is 122–168/100,000 (Landrum et al., 2012). Some populations acquire the disease more frequently. For example, SSTI rates of 4.15% have been reported for soldiers undergoing military training (Ellis et al., 2014). SSTI accounts for 14 million outpatient and emergency visits in the United States (Hersh et al., 2008). For pediatric SSTI cases, up to 72% of patients report recurrent disease within a year (Creech et al., 2015). *S. aureus* invasive disease can manifest as bacteremia, urinary tract infection, osteomyelitis, abscess formation, endocarditis, and/or sepsis (Lowy, 1998). Individuals at high risk for *S. aureus* infection include surgical patients, individuals with foreign body implants or low-birth-weight neonates, patients with indwelling catheters, endotracheal intubation, ventilator-assisted respiration, or immunosuppressive or cancer therapy, diabetics, end-stage renal disease patients, and nursing home residents (Spellberg and Daum, 2012). SSTI-associated hospitalization in the United States reached 358,212 in 2009, about half of all *S. aureus*–related hospitalizations, with an economic burden of USD 4.84 billion (Suaya et al., 2014). The prognosis of these infections is diminished by drug resistance (Chambers and DeLeo, 2009). Antibiotic-resistant strains, commonly designated MRSA (methicillin-resistant *S. aureus*), are resistant against many different drugs, and MRSA infection is associated with therapeutic failure (Chambers and DeLeo, 2009). The MRSA pandemic mandates antibiotic resistance testing for *S. aureus* isolates, antibiotic decolonization, and antibiotic prophylaxis for at-risk patient populations, which provides for further selection of antibiotic resistance (Chambers and DeLeo, 2009). Alternative strategies for reducing *S. aureus* disease are urgently needed; none would be more welcome than a preventive vaccine.

S. aureus genomics

Multilocus sequence typing (MLST) of single nucleotide variations of seven *S. aureus* housekeeping genes generates information about a strain's sequence type (ST; Chambers and DeLeo, 2009). STs that differ by single nucleotide changes at no more than three genes are grouped together as a clonal complex (CC). 11 CC types encompass >88% of all *S. aureus* isolates, and 5 CC types represent the human isolates in North America: CC1, CC5, CC8, CC30, and CC45 (Chambers and DeLeo, 2009). *spa* typing is based on the nucleotide sequence of the tandem repeats in the gene

Correspondence to Olaf Schneewind: oschnee@bsd.uchicago.edu

Abbreviations used: CC, clonal complex; ClfA, clumping factor A; Coa, coagulase; CP, capsular polysaccharide; IgBD, Ig-binding domain; IsdB, iron-regulated surface determinant B; MRSA, methicillin-resistant *S. aureus*; OPA, opsonophagocytic activity; SpA, staphylococcal protein A; SSTI, skin and soft tissue infection; ST, sequence type; vWbp, von-Willebrand factor–binding protein.

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for staphylococcal protein A (SpA; Shopsin et al., 1999). The tandem repeats vary in number and sequence, which can be exploited for epidemiological studies of outbreaks caused by specific STs. Whole-genome sequencing provided insights into the epidemiology and evolution of *S. aureus* (Lindsay, 2014). Over 10,000 yr, *S. aureus* evolved to colonize humans and their domesticated animals. All *S. aureus* isolates share a core genome enabling colonization and disease pathogenesis in their human hosts (McCarthy and Lindsay, 2010). Loss of the CRISPR-cas locus enabled bacteriophage-mediated acquisition of genomic islands for enhanced virulence, immune evasion, expanded host-range, and antibiotic resistance (McCarthy and Lindsay, 2010). CC75 is found in the Southwest Pacific and has been isolated from superficial skin lesions (Holt et al., 2011). CC75 retains the CRISPR-cas system, is endowed with *coa*, *spa*, and other core virulence genes but lacks the gene cluster for staphyloxanthin production (Holt et al., 2011). Staphyloxanthin is an important defense against phagocyte-derived reactive oxygen species and endows *S. aureus* colonies with their characteristic golden pigment (Pelz et al., 2005; Clauditz et al., 2006).

Whole-genome sequencing is used increasingly for epidemiological studies and provides important insights on genome content and variation. The *coa* gene, specifically the coding sequence for the D1-D2 domain of coagulase (Coa) involved in prothrombin binding (Friedrich et al., 2003), is the most variable sequence in the *S. aureus* genome (Watanabe et al., 2009). Most of the other genes involved in immune evasion and disease pathogenesis are not subject to significant sequence variation (Watanabe et al., 2009; McCarthy and Lindsay, 2010). Staphylococcal chromosome cassette *mec* (SCC*mec*) carries *mecA*, the PBP2A (penicillin-binding protein 2A) determinant for resistance against β-lactam antibiotics (Matsuhashi et al., 1986). Different variants of SCC*mec* are responsible for horizontal transmission of antibiotic resistance traits among different ST and CC types (Ito et al., 2001). Thus, *S. aureus* is a clonal pathogen, endowed with a broad spectrum of determinants that enable its virulence, immune evasion, and antibiotic resistance (Thammavongsa et al., 2015b).

Ghost of vaccines past

The success story of bacterial vaccines can be traced back to a handful of discoveries. Whole-cell preparations of bacteria grown in laboratory media, when injected into animals or humans, can elicit immune responses that are protective against naturally transmitted infectious diseases (Pasteur, 1881). To reduce vaccine-associated side effects, whole-cell vaccines can be heritably attenuated for virulence, while maintaining their ability to elicit protective immunity. For example, live-attenuated *Bacillus anthracis* has been used as a vaccine to protect animals and humans against anthrax disease (Sterne, 1946). Killed whole-cell preparations have also been used as vaccines in humans, for example, the whole-cell *Bordetella pertussis* vaccine for protection against whooping cough (Cherry, 1996). Whole-cell preparations of *S. aureus* have been tried in humans; however, these vaccines failed to demonstrate protective efficacy (Rogers and Melly, 1965). This is not entirely surprising. Unlike whooping cough or anthrax, prior infection does not elicit protective immunity against *S. aureus* recurrent disease (Rogers and Melly, 1965). Thus, neither immune responses of individuals with immunity nor animal models developing robust protection can be used as resources for *S. aureus* vaccine development.

The observation that some pathogens elaborate large capsular polysaccharides (CPs) providing for resistance to phagocytosis and promoting colonization of the human nasopharynx was another key discovery for vaccinology (Heidelberger and Avery, 1923). Capsules elicit poor antibody responses as major histocompatibility complexes cannot present polysaccharides as antigens to B cells or activate T cell help (Pollard et al., 2009). Chemical conjugation of polysaccharide to protein overcomes this deficit, eliciting robust antibody and B cell memory responses (Avery and Goebel, 1929). As demonstrated for the CP of *Haemophilus influenzae* type b, conjugate vaccine elicits antibodies that prevent nasopharyngeal colonization and infection, protecting vaccinated infants against bacteremia, pneumonia, meningitis, epiglottitis, and otitis media (Schneerson et al., 1980; Black et al., 1991). Capsule-conjugate technology has been expanded to generate vaccines against other pathogens, including *Neisseria meningitidis* (Frasch, 2005), *Streptococcus pneumoniae* (Black et al., 2000), and *Salmonella typhi* (Lin et al., 2001). The discovery of CP in *S. aureus* prompted the development of capsular-conjugate vaccines (Fattom et al., 1990). All clinical *S. aureus* isolates can be categorized as two CP types (CP5 and CP8) or capsule negative (CP^-; Fig. 1; Boyle-Vavra et al., 2015). CP5 and CP8 conjugates to *Pseudomonas aeruginosa* exotoxin A (StaphVAX, NABI Pharmaceuticals) elicit high-titer antibodies against both polysaccharides (Fattom et al., 2004). In efficacy trials with end-stage renal disease patients undergoing hemodialysis, capsule-specific antibodies failed to protect against *S. aureus* bacteremia (Shinefield et al., 2002).

Ghost of vaccines present

Some infectious diseases are caused by a single virulence trait in a pathogenic microbe. For example, the pathogenesis of diphtheria is critically dependent on diphtheria toxin (Behring, 1890; Collier, 2001). Adsorption of formalin-inactivated toxin to adjuvant (aluminum hydroxide) elicits strong antibody responses that neutralize diphtheria toxin and confer protective immunity (Glenny et al., 1931). This important advance accelerated the development of vaccines from murky whole-cell preparations into the realm of purified subunits, generating formulations with improved safety and carefully calibrated correlates of protection, i.e., neutralizing antibody titers. To identify subunit vaccines for *S. aureus*, a genomic approach was used to screen for antigens/epitopes of opsonophagocytic antibodies in human serum (Etz et al., 2002). This approach identified IsdB (iron-regulated surface determinant

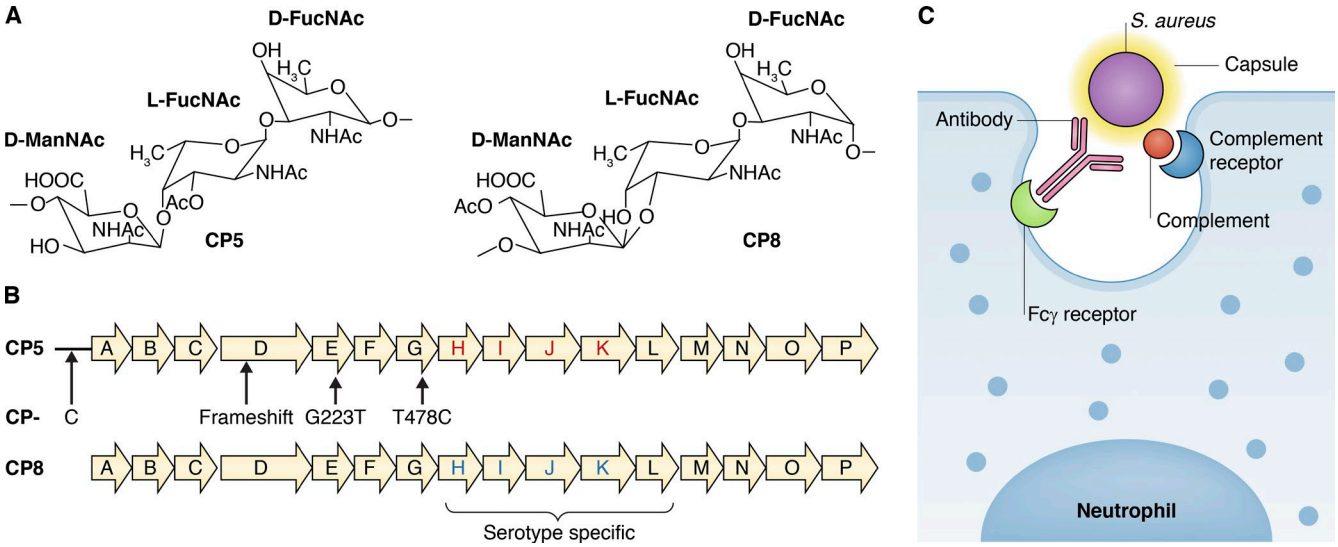

Figure 1. ***S. aureus* CPs.** (A) CP5 and CP8 have similar trisaccharide repeating structures that differ in glycosidic linkages and O-acetylation sites. (B) Genetic *cap* loci of *S. aureus* strains producing CP5 and CP8. Genes printed in color are responsible for the synthesis of serotype-specific modifications of CP. The diagram reveals the position of mutations in CP^- strains of the *S. aureus* USA300 and USA500 lineages (Boyle-Vavra et al., 2015). (C) Diagram illustrating the hypothesis that capsule-specific antibody may induce OPA, inducing uptake of *S. aureus* by neutrophils via FcγR or complement receptors.

B), a heme-iron scavenging surface protein contributing to the pathogenesis of *S. aureus* infections in animal models (Fig. 2; Mazmanian et al., 2003; Torres et al., 2006). Purified IsdB subunit (V710 vaccine) elicits antibodies that block heme-iron scavenging and provide partial protection against *S. aureus* bacteremia in preclinical models (Kim et al., 2010b). Other studies proposed that IsdB-specific antibodies may also promote opsonophagocytosis of *S. aureus* (Kuklin et al., 2006; Brown et al., 2009). Nonetheless, in a clinical trial with 7,045 thoracic surgery patients at risk for *S. aureus* infection, V710 not only failed to provide protection but also increased the risk for a fatal outcome of *S. aureus* bacteremia in vaccine recipients fivefold over the control cohort (Fowler et al., 2013). The molecular basis for the increased risk of fatal outcomes in V710 vaccine recipients is not known. Nonetheless, the V710 safety signal elevated regulatory awareness for the need to probe *S. aureus* vaccine safety in different patient populations.

Ghost of vaccines future

Pfizer advanced SA4Ag vaccine toward phase IIb trials in patients receiving posterior instrumented spinal fusion with a projected enrollment of 2,600 (Scully et al., 2014). Similar to other surgical procedures, these patients are at risk of *S. aureus* wound infection and bacteremia (Schuster et al., 2010). SA4Ag encompasses CP5 and CP8 conjugates to genetically inactivated diphtheria toxin (CRM197) in addition to two subunits, clumping factor A (ClfA), a fibrinogen-binding protein, and manganese transport protein C (MntC), which imports manganese from host environments for bacterial superoxide dismutase (SOD) activity (Fig. 3; Anderson et al., 2012). The trial's design appears to be based on the hypothesis that multiple antigens may elicit increased protection over single antigens. However, among FDA licensed vaccines, there is as of yet no precedence for the conjecture that multiple antigens are indeed better than one. Large clinical trials are of course time and resource consuming, and companies typically do not engage in reiterative trials to improve vaccine performance in pursuit of efficacy testing and licensure. Because of a string of clinical trial failures with vaccines or antibodies against *S. aureus* vaccines, pharmaceutical companies are hesitant to launch new clinical efficacy trials for *S. aureus* vaccines until the results of ongoing trials are known. Thus, in spite of a large unmet clinical need, progress toward *S. aureus* vaccines remains slow.

Clinical trial designs

Past and current clinical trials for *S. aureus* vaccines targeted individuals at high risk of infection. Target populations include elderly who are often afflicted by comorbidities that impact the immune system and susceptibility toward *S. aureus*, for example, diabetes and/or renal failure, deep surgical wounds, prosthetic implants, and intravenous catheters (Spellberg and Daum, 2012). In contrast, successful vaccines have been administered to large cohorts of healthy subjects, typically infants or children, to prevent childhood diseases. As an added benefit, successful vaccines reduce the colonization of humans with the microbial target. For example, immunization with pneumococcal polysaccharide-conjugate vaccine diminishes nasopharyngeal colonization with vaccine serotypes, which in time triggers replacement with nonvaccine serotypes requiring serotype expansions of conjugate vaccines (Geno et al., 2015). Future clinical trials of pneumococcal

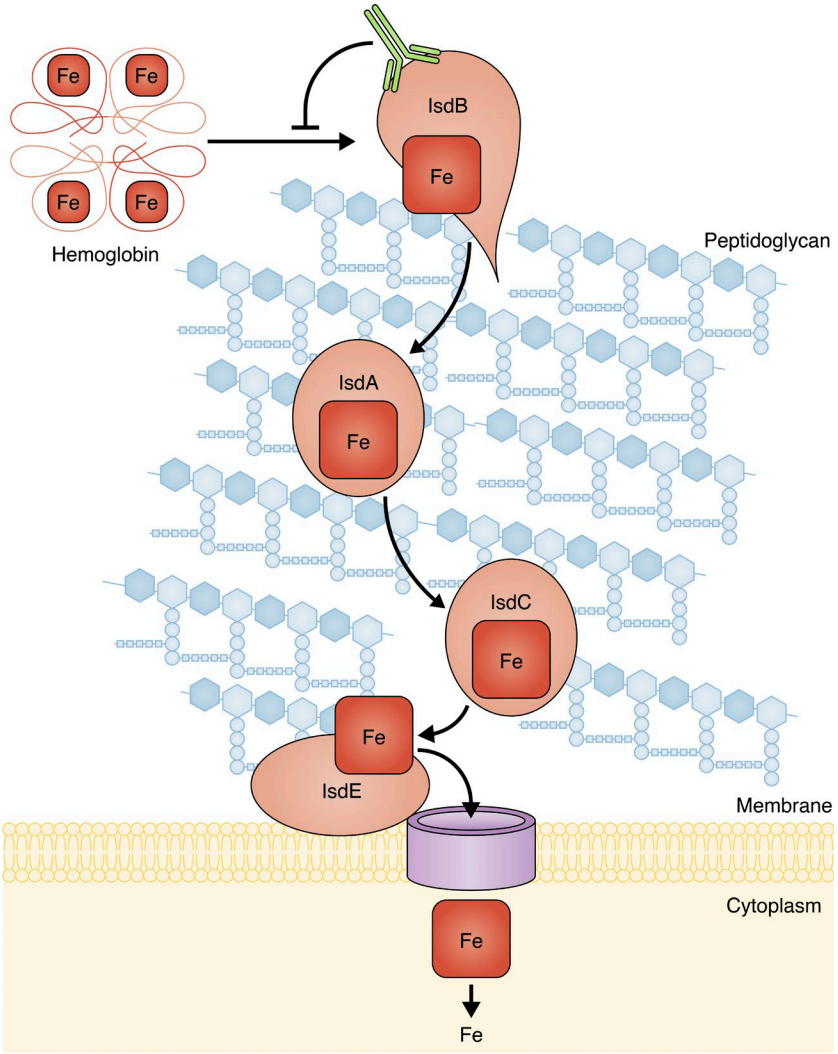

Figure 2. **IsdB surface protein and heme–iron scavenging of *S. aureus*.** Diagram illustrating the physiological role of IsdB in scavenging heme-iron from host hemoglobin and transferring this nutrient across the bacterial envelope, followed by iron release from heme in the bacterial cytoplasm. Antibodies against IsdB are thought to block heme-iron scavenging during infection (Kim et al., 2010b).

vaccines may well use nasal colonization data as endpoints for vaccine efficacy and licensing (Auranen et al., 2013). Thus far, a single pilot study has examined nasal colonization as endpoint for *S. aureus* vaccine efficacy studies. Healthy human volunteers (n = 88) were examined for nasal colonization 2 and 1 wk before vaccination with StaphVAX and again 6 wk after vaccine administration (Creech et al., 2009). Although StaphVAX elicited robust antibody responses against CP5 and CP8, the vaccine did not affect nasal colonization (Creech et al., 2009). These results do not preclude the possibility that other vaccines may impact *S. aureus* colonization and, consequently, the incidence of SSTI.

Lack of correlates for protective immunity

In cases where microbes rely on a single secreted toxin for disease pathogenesis, protective immunity is easily quantified as the concentration of antibody with toxin-neutralizing activity. For the Hib vaccine, the serum concentration of polysaccharide-specific antibody, as measured by ELISA, has been correlated with protection against *H. influenzae* type b bacteremia (≥0.15 mcg/ml) and nasopharyngeal colonization (≥5 mcg/ml; Plotkin, 2008). Correlates of protection for pneumococcal vaccines in children and the elderly are measured as the concentration of capsular antibody (0.2–0.35 mcg/ml) able to induce opsonophagocytosis of corresponding serotype *S. pneumoniae* by HL60 cells, i.e., human promyelocytic leukemia cells that can be differentiated into functional granulocytes (Plotkin, 2008). Finally, *N. meningitidis* polysaccharide-specific antibodies are bactericidal as the result of complement activation; protection against colonization and bacteremia occurs when serum dilutions 1/8 or higher display bactericidal activity (Goldschneider et al., 1969). Among healthy adults, the concentration of antibodies against *S. aureus* CP5 and CP8 is 10–15 mcg/ml, which was increased by 10–20-fold after immunization with capsular-conjugate vaccine, yielding geometric means of 230 and 206 mcg/ml, respectively (Fattom et al., 2004). CP-specific antibodies are maintained at high levels over 6 mo or longer. Serum concentrations of 80 mcg/ml were sufficient to promote some opsonophagocytic killing of *S. aureus* by HL60 cells at a mul-

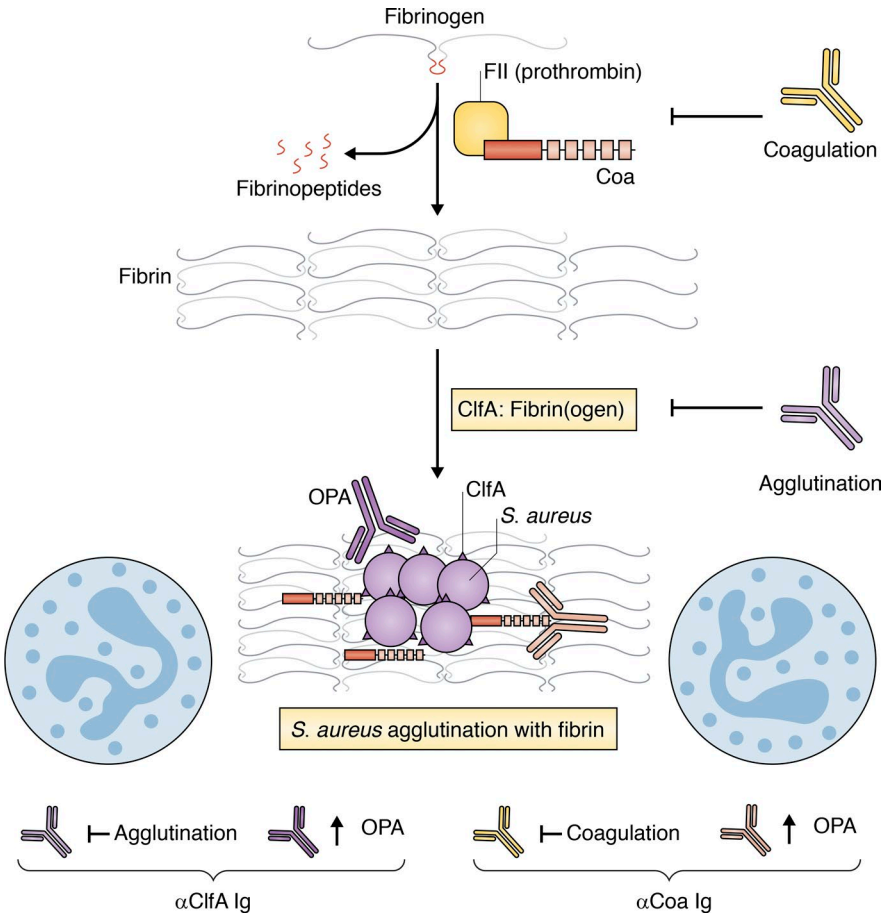

Figure 3. ***S. aureus* agglutination with fibrin as a defense against phagocytosis.** Diagram illustrating the agglutination pathway. Staphylococcal Coa, a secreted protein, associates with factor II (FII, prothrombin). The resulting staphylothrombin complex removes fibrinopeptides A and B off fibrinogen to generate fibrin cables. ClfA, a staphylococcal surface protein, binds to fibrinogen and fibrin, thereby coating *S. aureus* with a fibrin shield for protection against host neutrophils. Antibodies against Coa or ClfA may prevent the association with FII and fibrin, respectively, thereby blocking staphylococcal agglutination and/or inducing OPA.

tiplicity of infection of 1:100 (bacteria/HL60 cells; Fattom et al., 2004). Nonetheless, CP-specific antibody–induced opsonophagocytic activity (OPA) in HL60 is not a correlate of protection as high levels of antibodies are not known to prevent *S. aureus* colonization or invasive disease (Shinefield and Black, 2005; Creech et al., 2009). Of note, OPA assays with HL60 cells lack hemostasis factors (prothrombin and fibrinogen) that represent key immune evasive strategies of *S. aureus* (Thammavongsa et al., 2015a). Thus, in contrast to *S. pneumoniae* isolates, which do not manipulate host hemostasis, the HL60 cell assay for OPA is unlikely to predict protective antigens OPA for *S. aureus*.

Factors manipulating host hemostasis

The hallmark of all *S. aureus* isolates is their ability to coagulate human and animal plasma, which is based on two secreted factors, Coa and von-Willebrand factor–binding protein (vWbp; Fig. 3; Cheng et al., 2011). Both polypeptides bind prothrombin (FII) via their D1-D2 domains and non-proteolytically activate the zymogen to cleave fibrinopeptides (A and B) off fibrinogen, thereby promoting assembly of fibrin cables (Friedrich et al., 2003). Of note, Coa and vWbp cleave fibrinogen but not the regulatory component targeted by proteolytically activated prothrombin during hemostasis (McAdow et al., 2012b). *S. aureus* deploys surface proteins, for example ClfA and fibronectin-binding protein A (FnBPA), to capture Coa-derived fibrin cables on its surface (McAdow et al., 2011). The resulting fibrin shield protects staphylococci from OPA in human and animal blood and also promotes the formation of abscesses in infected tissues (Cheng et al., 2010). Neutralizing antibodies against Coa have been used for the serotyping of *S. aureus* isolates (Watanabe et al., 2009). When examined in the mouse bacteremia model, antibodies against the D1-D2 domain of Coa provide type-specific protection against *S. aureus* (McAdow et al., 2012a). As Coa sequence/sero-types correlate with ST/CC types, such antibodies may be valuable for protection of humans against blood-borne infection caused by *S. aureus*. Irrespective of serotype, all Coa STs harbor C-terminal repeats of a 27-residue peptide, which binds fibrinogen and enables staphylococci to polymerize fibrin cables on the bacterial surface (Thomer et al., 2016). Of note, antibodies against the repeat domain promote OPA of *S. aureus* in blood and provide broad protection against mouse bloodstream infection when challenged with different ST/CC types (Thomer et al., 2016). vWbp–prothrombin complexes display slower kinetic rates for fibrinogen cleavage than Coa–prothrombin, and vWbp also supports the pathogenesis of *S. aureus* bloodstream infection (Kroh et al., 2009; Cheng

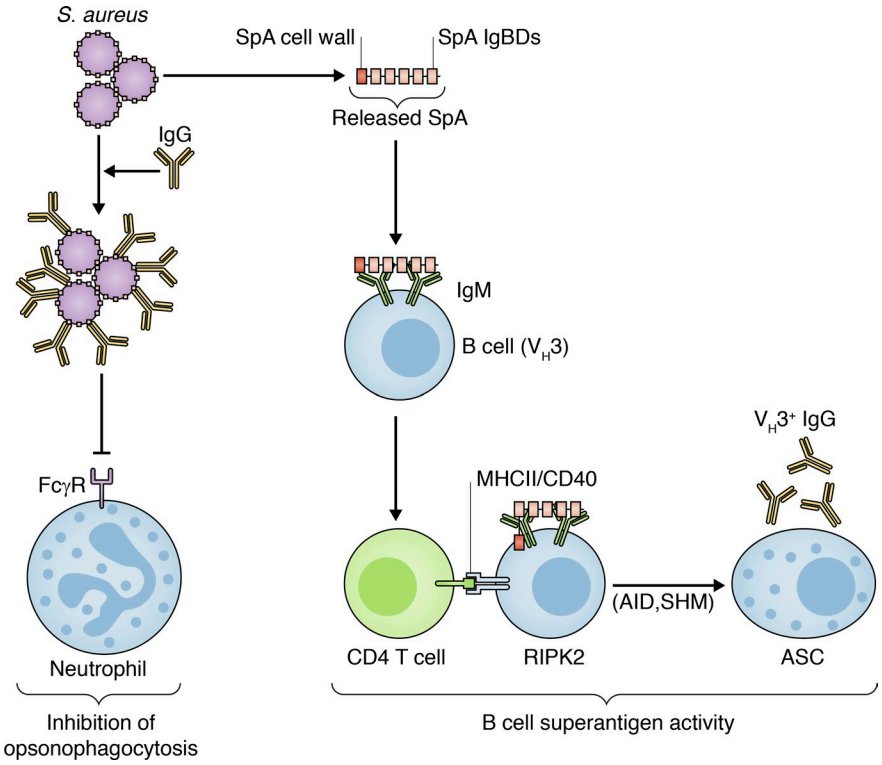

Figure 4. **Immune evasive attributes of SpA.** SpA coats the surface of *S. aureus* and binds the Fcγ domain of antibodies to block their effector functions and inhibit opsonophagocytosis. Cell wall–anchored SpA is released from the bacterial surface by murein hydrolases and cross-links B cell receptors of V_H3 clonal B lymphocytes. SpA induces B cell proliferation and maturation into antibody-secreting cells (ASC) via a pathway requiring CD4 T cells, MHC class II activation, activation-induced cytidine deamination (AID), and somatic hypermutation (SHM) for the secretion of mature antibodies. SpA induces the secretion V_H3 clonal antibodies that are nonreactive to staphylococcal antigens. The B cell superantigen activity is dependent on the peptidoglycan modification of SpA and on RIPK2 signaling by immune cells.

et al., 2010). vWbp plays a unique role during the assembly of abscess lesions. Although Coa–prothrombin generates fibrin shields surrounding staphylococcal abscess communities, vWbp–prothrombin establishes peripheral fibrin deposits demarcating infectious lesions from healthy tissue (Cheng et al., 2010; Guggenberger et al., 2012). Similar to Coa, vWbp is also sequence variable, and antibodies against both proteins provide protection against renal abscess formation in mice (Cheng et al., 2010). vWbp cannot associate with bovine prothrombin, and *S. aureus* strains causing invasive disease in these animals acquire a pathogenicity island–encoded vWbp homologue that activates bovine prothrombin (Viana et al., 2010). We propose that the study of *S. aureus* OPA in human blood or HL60 cells incubated with human hemostasis factors may lead to the establishment of correlates for protective immunity.

Protein A

All clinical isolates express SpA, a surface protein comprised of up to five Ig-binding domains (IgBDs), tandem repeats of an 8-residue peptide and a C-terminal LPXTG sorting motif for cell wall anchoring (Kim et al., 2012; Votintseva et al., 2014). SpA binds to the Fc-γ domain of IgG and blocks Ig effector functions toward OPA (Fig. 4; Forsgren and Quie, 1974). The IgBDs also bind to the variant heavy chains of V_H3 clonal Ig, cross-linking B cell receptors (IgM) and promoting B lymphocyte proliferation (Forsgren et al., 1976; Silverman and Goodyear, 2006). During *S. aureus* infection in humans and in mice, SpA promotes increases in V_H3 clonal serum IgM and IgG (Pauli et al., 2014; Kim et al., 2016). These antibodies, however, are not specific for *S. aureus* and do not provide protective benefits. In contrast, SpA variants that cannot bind Ig yet retain antigenic structure elicit pathogen-specific antibody responses that provide protection in preclinical disease models (Falugi et al., 2013). Similar effects can be achieved by immunization with nontoxigenic SpA subunit, designated SpA_{KKAA}, which elicits SpA neutralizing antibodies not found during *S. aureus* infection (Kim et al., 2010a). B cell superantigen activity relies not only on SpA IgBDs but also on its C-terminal peptidoglycan linkage and release from the bacterial envelope (Kim et al., 2016). NOD1/NOD2 (nucleotide oligomerization domain 1 and 2) signaling via RIPK2 (receptor-interacting serine/threonine protein kinase 2) and CD4 T helper cells are other essential components of the *S. aureus* immune evasion pathway (Kim et al., 2016). SpA expression may be required for *S. aureus* survival and colonization of the human nares (Cole et al., 2016). However, it is not known whether SpA-specific antibodies impact *S. aureus* nasal colonization or the serum antibody responses associated with colonization.

Concluding remarks

Vaccine trials in the past have failed to protect patients at elevated risk of *S. aureus* infection. Although hospital patients enable the design of small-sized clinical trials, it is not clear that such populations can be protected in future vaccine trials. *S. aureus* colonization and SSTI have not yet been rigorously examined as endpoints in large clinical trials. Studies on the prevention of disease in healthy individuals may offer

new opportunities for the development of *S. aureus* vaccines. Unlike CPs of other bacterial pathogens, the *S. aureus* type 5/8 CP is likely not essential for colonization or invasive disease and may not function as protective antigen. Targeting immune evasive factors of *S. aureus* for vaccine development holds promise, at last in preclinical studies. Nonetheless, correlates of immunity and mechanisms for phagocyte-mediated killing of *S. aureus* remain to be established and are likely essential for the development of a successful vaccine.

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