Pathogenesis of *Staphylococcus aureus* Bloodstream Infections

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Abstract

Staphylococcus aureus , a Gram-positive bacterium colonizing nares, skin, and the gastrointestinal tract, frequently invades the skin, soft tissues, and bloodstreams of humans. Even with surgical and antibiotic therapy, bloodstream infections are associated with significant mortality. The secretion of coagulases, proteins that associate with and activate the host hemostatic factor prothrombin, and the bacterial surface display of agglutinins, proteins that bind polymerized fibrin, are key virulence strategies for the pathogenesis of *S. aureus* bloodstream infections, which culminate in the establishment of abscess lesions. Pathogen-controlled processes, involving a wide spectrum of secreted factors, are responsible for the recruitment and destruction of immune cells, transforming abscess lesions into purulent exudate, with which staphylococci disseminate to produce new infectious lesions or to infect new hosts. Research on *S. aureus* bloodstream infections is a frontier for the characterization of protective vaccine antigens and the development of immune therapeutics aiming to prevent disease or improve outcomes.

Keywords

coagulation; agglutination; immune evasion; abscess formation; vaccine

INTRODUCTION

The Gram-positive microbe *Staphylococcus aureus* stably colonizes the nares, the skin, and/or the perineum of approximately one-third of the human population, whereas another third is colonized intermittently (1). *S. aureus* is also an invasive pathogen and frequent cause of skin and soft tissue as well as bloodstream infections (2). Once the pathogen enters the bloodstream, *S. aureus* replicates and disseminates to many different sites, causing severe disease manifestations such as sepsis, infective endocarditis, and deep-seated abscesses in virtually every organ tissue (2). *S. aureus* has evolved resistance mechanisms against antibiotics. Vancomycin, daptomycin, clindamycin, and linezolid are approved for the treatment of infections with antibiotic-resistant strains [MRSA (methicillin-resistant *S. aureus*)]; however, MRSA isolates develop resistance even against last resort therapeutics, and resistance is associated with therapeutic failure and increased mortality (2, 3). There is a clear medical need for vaccines and immune therapeutics that can address the public health

crisis of MRSA infections. Although vaccines and antibody therapeutics targeting *S. aureus* capsular polysaccharides, surface proteins, and toxins are being developed, thus far such regimens have not reached the endpoints of clinical efficacy trials (4). This article summarizes what is known about the pathogenesis of *S. aureus* bloodstream infections— information that may be useful for the development of vaccines or immune therapeutics.

THE PATHOGEN

S. aureus

S. aureus was first isolated from the pus of surgical wounds by Alexander Ogston, who initially referred to the bacteria as micrococci (5). Their characteristic appearance—grape-like clusters (*staphyle* in Greek) of sphere-shaped bacteria—prompted Ogston to name the organisms staphylococci, which distinguishes them from chain-forming streptococci, also associated with surgical wound infections. Ogston further reported that injection of staphylococci into the subcutaneous tissue of experimental guinea pigs and mice produced abscess lesions (6).

Rosenbach differentiated staphylococci isolated from humans based on the pigmentation of their colonies, proposing the nomenclature *Staphylococcus aureus* and *Staphylococcus albus* for yellow and white colonies, respectively (7). The latter species is now designated *Staphylococcus epidermidis.* Staphyloxanthin, a membrane-bound carotenoid produced by *S. aureus*, is responsible for the yellow pigmentation of colonies. Pigment production scavenges reactive oxygen species and protects *S. aureus* from phagocytic killing (8). The ability to clot human and animal blood or plasma is a hallmark of *S. aureus* and is mediated by two secreted products, coagulase (Coa) and von Willebrand factor–binding protein (vWbp); *S. epidermidis* is Coa negative (9). Several other staphylococcal species are also classified as Coa positive, including *S. delphini*, *S. intermedius*, and *S. pseudintermedius*, species that have adapted to various hosts (mink, fox, pigeon, and dog) but do not colonize humans (10).

Clinical Burden of *S. aureus* Infections

In the United States, the annual burden of *S. aureus* diseases requiring medical treatment includes more than one million skin and soft tissue infections (SSTIs) with and without deep-seated abscess lesions; 490,000 hospitalizations; 93,000 cases of bacteremia; and 35,000 cases of sepsis and/or endocarditis (11). An annual mortality rate of more than 20,000 is attributed to *S. aureus* infection. Of particular concern are patients with recurrent SSTIs or bacteremia (12). Recurrent infection leads to invasive *S. aureus* disease and sepsis. Specific patient populations are at elevated risk of healthcare associated (HA) *S. aureus* infection: very low birth-weight neonates and patients with indwelling catheters, endotracheal intubation, medical implantation of foreign bodies, trauma, surgical procedures, hemodialysis, peritoneal dialysis, diabetes and who have undergone immunosuppressive or cancer therapy (12). Clinical use of antibiotics led to the selection of drug-resistant strains, designated MRSA for methicillin-resistant *S. aureus*. Acquisition of a specific penicillin-binding protein, PBP2a, that cannot be inhibited by penicillinase-resistant β-lactams (methicillin or oxacillin) is the molecular basis for broad-spectrum resistance

against this class of compounds (13, 14). MRSA isolates account for >60% of clinical *S. aureus* infections in Japan, >50% in Italy and Portugal, and >30% in the United States (15). MRSA isolates are generally multidrug resistant and require treatment with vancomycin, clindamycin, linezolid, or daptomycin (3). The use of vancomycin as the antibiotic of last resort against MRSA has selected for the emergence of vancomycin-resistant *S. aureus* and is also responsible for the frequent occurrence of strains of with intermediate resistance (reviewed in 2).

Skin and Soft Tissue Infections

Breaches in skin barrier function occur following trauma and surgical procedures and favor entry of *S. aureus* into subcutaneous tissues. Nevertheless, *S. aureus* infection can occur at sites without apparent breaches, for example at hair follicles (folliculitis), as bullous or superficial lesions (impetigo), or as deep or confluent abscesses (furuncles or carbuncles, respectively) (2). All of these infections are referred to as SSTIs and manifest as purulent exudates draining from the infectious site. Approximately 20% of patients with SSTIs undergoing antibiotic therapy develop recurrent infections with the same strain (16). Thus, prior infection with *S. aureus* is not known to generate a protective immune response.

Studies in mice have revealed pathophysiological features associated with *S. aureus* SSTIs (for an extensive review, see 17). Within 24 h following subcutaneous inoculation of bacteria, a large inflammatory response is observed with indurated swelling at the site of infection. These lesions expand in size over 5–7 days and then resolve over the next 7–9 days (18). Dermonecrosis is often observed with subcutaneous abscess lesions, heals at a similar rate as the lesion resolves, and is caused by secreted products, α-hemolysin (α-toxin, Hla) (18), and phenol-soluble modulins (PSMs) (19). Some MRSA strains express the bacteriophage-encoded bicomponent Panton-Valentine leucocidin (PVL-S and PVL-FS), which binds to human complement receptors C5aR and C5L2 and mediates lysis of leukocytes (but not of murine immune cells) (20). PVL expression is thought to aggravate the pathology of *S. aureus* infections in humans (21). PSMs contribute to the pathogenesis of mouse skin infections and elicit proinflammatory responses by interacting with formyl peptide receptor 2 of immune cells and by activating, attracting, and lysing neutrophils (22). In contrast to immune-competent animals, leukopenic mice develop systemic infections with rapidly lethal outcomes following skin inoculation of *S. aureus* (23). In humans, leukopenia and hereditary defects in the NADPH oxidase or in the respiratory burst of myeloid cells are associated with increased susceptibility toward *S. aureus* SSTIs (24). Humans with abnormal T cell function (including HIV/AIDS patients), atopic dermatitis, and hyper-IgE syndrome display increased susceptibility toward *S. aureus* infection (25–27). Increased severity of skin infections has been observed in MyD88- and interleukin (IL)-1R-deficient mice, and to a lesser extent in Toll-like receptor (TLR)2-deficient mice. Compared with wild-type mice, MyD88-, IL-1R-, and TLR2-knockout animals develop larger skin lesions and elevated burden of staphylococci, which correlate with the decreased recruitment of neutrophils to the site of infection (28). Similar phenotypes were observed among mice lacking dendritic epidermal T cells or IL-17R (29). IL-17-producing T (T_H17) lymphocytes play an important role in controlling *S. aureus* cutaneous infection by recruiting neutrophils to the site of infection. A growing body of evidence implicates T_H17 cells as critically important for

protection against *S. aureus* skin and lung infections; however, $T_H17/IL-17$ responses may be less important for *S. aureus* infections in other tissues (30).

Bloodstream Infections

S. aureus is a leading cause of bacteremia, with an annual incidence of 4.3 to 38.2 per 100,000 person-years in the United States (31). The 30-day all-cause mortality of *S. aureus* bacteremia is 20% and has not changed since the 1990s (32). Based on its onset, bacteremia due to *S. aureus* can be classified into three categories: HA with hospital onset (33); HA with community onset (infection in an outpatient who has had recent, extensive contact with the health-care system); and community acquired (CA). In a prospective cohort study of 504 bloodstream infections at an academic center and two community hospitals in 2000, 35% were nosocomial, 37% were HA with community onset, and the remaining 28% were CA infections (34). MRSA predominated in nosocomial infections and in HA infections with community onset (61% and 52%, respectively); the MRSA burden was lower in patients with CA infections (14%).

The most prominent risk factor for invasive *S. aureus* infection and bacteremia is prosthetic devices, including central venous catheters, surgically implanted materials, and orthopedic prostheses (35). These devices serve as a direct conduit into the intravascular space, allowing *S. aureus* access to the bloodstream. Furthermore, intravenous drug use and underlying medical comorbidities such as diabetes, immunosuppressive therapy, and malignancy predispose individuals to *S. aureus* bacteremia (36).

STAPHYLOCOCCAL DEFENSES AGAINST COMPLEMENT AND PHAGOCYTES

S. aureus has evolved an arsenal of factors to evade innate immunity. Neutrophils represent the most abundant polymorphonuclear leukocyte population in blood and are a crucial defense against *S. aureus* (37). The pathogen, in turn, targets all stages of neutrophil recruitment as well as neutrophil effector functions. Staphylococci keep neutrophils from migrating toward the site of infection by impeding chemotaxis via the secretion of staphylococcal superantigen-like 5 and 10 (SSL5 and SSL10); chemotaxis inhibitory protein of *S. aureus* (CHIPS); and its homologs, formyl peptide receptor-like inhibitory proteins (FLIPr and FLIPr-like) (38–40). Further, SSL5 prevents neutrophils from rolling on endothelial cells, a crucial step in their recruitment process (41). This interference is aided by staphylococcal secretion of extracellular adherence protein, which blocks adherence of neutrophil ligands to cognate endothelial adhesion receptors (42). Interestingly, *S. aureus* can also stimulate neutrophil chemotaxis via the secretion of formylated PSMs (43).

Neutrophil phagocytosis and microbial clearance are facilitated when target microbes are opsonized by components of the complement system and/or immunoglobulins (Igs) (44). Complement can rapidly recognize and opsonize bacteria or directly kill bacteria via the formation of membrane attack complexes (45). Recognition of nonself and activation of the complement cascade occur by any one of three independent pathways; however, *S. aureus* diminishes or delays opsonization by targeting a universal step of complement activation:

factor C3 and its activation complexes, also designated C3 convertases (44). *S. aureus* blocks C3 convertases via staphylococcal complement inhibitor (SCIN) or the SCIN-B and SCIN-C homologs or with extracellular fibrinogen binding protein and its homolog, extracellular complement binding protein (46). *S. aureus* secretes aureolysin, a metalloprotease that cleaves and inactivates C3 (47), and recruits complement inhibitory factors H and I via surface-anchored proteins SdrE and clumping factor A (ClfA), another pathway promoting C3 cleavage (48). Staphylococci target complement factor C5 via the secretion of SSL7 (49). To prevent opsonization by Ig, *S. aureus* evolved Ig-binding factors, such as staphylococcal protein A (SpA), staphylococcal binder of Ig, and SSL10; each of these factors blocks the Fcγ effector domain of opsonizing antibodies (50–52). Staphylococcal binder of Ig also targets C3 via factor H (52). Staphylokinase (Sak) generates enzymatically active plasmin that cleaves fibrin, Ig, and C3 deposited on the bacterial surface (53). Staphylococci have several means to generate a protective coat composed of polysaccharide capsule or fibrin; such coatings impede phagocytic uptake of staphylococci by neutrophils or macrophages (54, 55) (Figure 1).

Neutrophils kill bacteria via phagocytosis, generating reactive oxygen and reactive nitrogen species and releasing antibacterial enzymes or peptide defensins from granules (37). Alternatively, neutrophils may release NETs (neutrophil extracellular traps), which are composed of neutrophil DNA, histones, antimicrobial peptides, and neutrophil proteases (56). *S. aureus* evades reactive oxygen species via staphyloxanthin, its yellow pigment and antioxidant, catalase, and other detoxifying enzymes that convert hydrogen peroxide to oxygen and water (57, 58). Reactive nitrogen species are detoxified by flavohemoglobin, a potent scavenger of host NO• (59). *S. aureus* metabolically adapts to circumvent the metabolic consequences of nitrosative stress, using lactate dehydrogenase to maintain redox hemostasis (60). Staphylococci fortify their cell wall envelope against muralytic enzymes and antimicrobial peptides using chemical modifications of peptidoglycan and synthesis of secondary cell wall polymers, including WTA and LTA (61, 62). Secreted factors such as Sak and aureolysin inactivate antimicrobial peptides by direct binding or proteolytic cleavage (63, 64). Finally, *S. aureus* secretes nuclease to degrade NETs, not only to escape DNA entrapment and peptide defensins, but also to generate deoxyadenosine, which triggers caspase-3-mediated death of immune cells (65, 66). Generation of deoxyadenosine and associated immune cell killing depend on staphylococcal adenosine synthase A, a surface protein that also accepts adenine nucleotides as substrates for the synthesis of adenosine and immunosuppressive signaling via adenosine receptors (66, 67).

Beyond provisions for escape from innate immunity to ensure staphylococcal survival in host tissues, *S. aureus* secretes toxins to disrupt epithelial or endothelial surfaces and to trigger lysis of immune cells (20). The most prominent cytolytic toxin is Hla, a pore-forming toxin with the capacity of lysing epithelial, endothelial as well as immune cells (69). Hla-mediated insults occur in a manner correlated with tissue-specific expression of ADAM10 (A-disintegrin and metalloprotease 10), the host receptor for Hla (69). Following Hla binding to ADAM10, the toxin assembles into a heptameric pore structure (70), causing cellular injury and activating signaling cascades that perturb the integrity of epithelial and endothelial surfaces (71). *S. aureus* strains produce three or more leukocidins, bi-component pore-forming toxins, where HlgAB, HlgCB, and LukAB (also known as LukGH) represent

the core genome encoded virulence factors (72). Some *S. aureus* strains express one or two additional bi-component pore forming toxins, PVL and LukED (also known as LukMF) (72). Bi-component pore-forming toxins associate with specific receptors on myeloid cells, including complement and chemokine receptors or CD11b integrin (72). PSMs, small peptides with amino-terminal formyl groups that are secreted via a signal peptide independent mechanism (73), target eukaryotic cells, including immune cells, in a receptor-independent process and trigger their lysis (19); these toxic attributes depend on the unique charge distribution of individual PSMs (74).

STAPHYLOCOCCAL INTERFERENCE WITH HEMOSTASIS

S. aureus clots human or animal blood even in the presence of coagulation inhibitors such as warfarin, heparin, hirudin or calcium chelators (75). Staphylococcal coagulation is activated by two secreted proteins, Coa and vWbp, that bind prothrombin i.e., factor II of the blood coagulation pathway to generate enzymatically active staphylothrombin complexes (76, 77) (Figure 2*a*). Under physiological conditions, prothrombin activation requires cleavage by the prothrombinase complex composed of factors Va/Xa of the extrinsic blood coagulation pathway, also known as tissue factor pathway. Vascular damage initiates the tissue factor pathway and leads to the rapid and highly localized activation of a series of serine proteases that culminate with the accumulation of factors Va/Xa (78) (Figure 2*a*). Both thrombin and staphylothrombin complexes cleave the fibrinopeptides A and B off the fibrinogen α- and β-chains to generate fibrin (76, 77, 79). Fibrinogen is a soluble, 340-kDa dimer of trimers (α-, β-, and γ-chains), crosslinked by disulfides (79). When stripped of its fibrinopeptides, fibrinogen self-assembles into polymer cables to form a fibrin clot (79) that is further stabilized by factor XIIIa, a transglutaminase that crosslinks glutamine residues on one fibrin molecule to lysine residues of another. Factor XIIIa is itself the thrombin-cleaved product of the factor XIII zymogen (A_2B_2) (80).

Coa and vWbp are mosaic proteins made up of N-terminal D1 and D2 domains (D1–D2) that enable the association with and activation of prothrombin, whereas the C-terminal domains of the two molecules are distinct (81–83). A linker (L) domain connects the D1–D2 of Coa with the C-terminal repeat (R) region, composed of tandem repeats of a 27-residue peptide that bind fibrinogen (76) (Figure 2*b*). The C-terminal domain of vWbp includes a binding site for von Willebrand factor (vWF) (84) (Figure 2*b*). The vWbp-vWF interaction was identified via phage-display for recombinant vWbp (84); its biological significance remains unclear. The C-terminal part of vWbp recruits and activates factor XIII in a nonproteolytic manner and also binds to fibronectin (85) (Figure 2*b*). The crystal structure of the enzymatically active Coa·prothrombin complex revealed binding of the Coa D1–D2 to prothrombin and insertion of the Ile^1-Val^2 N terminus of Coa into the Ile^{16} pocket of prothrombin, inducing a functional active site in the zymogen through conformational change (76). Although vWbp·prothrombin complexes display slower enzyme kinetics than Coa·prothrombin, vWbp activates prothrombin by a similar mechanism (86). Staphylothrombins cleave the A and B fibrinopeptides of fibrinogen but do not cut any of the other substrates of thrombin. Thus, *S. aureus*, via the secretion of Coa and vWbp, usurps the zymogen of one clotting factor (prothrombin) to modify the coagulation cascade of human

blood, thereby causing exuberant, uncontrolled polymerization of fibrin without activation of other clotting and inflammatory factors.

Staphylococcal coagulation itself is part of a reaction known as agglutination, i.e., the formation of a network of staphylococci with fibrin, which can be visualized by inoculating *S. aureus* into plasma and observing the assembly of large aggregates of bacteria (87). A related phenomenon is observed when staphylococci are mixed with purified fibrinogen, which causes smaller clumps of bacteria; however, bona fide agglutination requires the host factors prothrombin and fibrinogen (88) (Figure 1). In addition to Coa- and vWbp-mediated prothrombin activation, staphylococcal agglutination is dependent on agglutinins, sortase-anchored surface proteins such as ClfA or fibronectin-binding protein A (FnBPA), which bind to the D-domain of fibrin (87) (Figure 2*a*). ClfA and FnBPA bind to the C terminus of the fibrinogen γ-chain, capping staphylothrombin-polymerized fibrin cables and tethering these cables to the bacterial surface (87, 89). ClfA was initially identified as the staphylococcal factor required for bacterial clumping with soluble fibrinogen (90, 91). Recent analyses of the molecular mechanism of agglutination revealed the concerted action of Coa, vWbp, and ClfA in the pathogenesis of bloodstream infection (87). In contrast to mutants lacking any one gene (*coa*, *vwb*, or *clfA*), which displayed reductions in virulence, a strain with all three genes deleted was avirulent in a mouse model of bloodstream infection, suggesting a synergistic mode of action between staphylococcal factors for hemostatic interference (87). Of note, ClfA and other *S. aureus* surface proteins with C-terminal serine-aspartate (SD) repeats are *N*-acetylglucosaminylated, posttranslational modifications that contribute to *S. aureus* agglutination in animal or human blood (92). The molecular mechanism whereby *N*-acetylglucosaminylation of SD repeat proteins contributes to staphylococcal agglutination is not known.

The product of agglutination endows *S. aureus* with a fibrin shield, effectively protecting bacteria from uptake by phagocytes. Of note, the coagulases fulfill nonredundant roles in establishing the protective fibrin barrier: vWbp has been shown to recruit factor XIII through its interaction with prothrombin and fibrinogen, leading to the fortification of the nascent fibrin clot with covalent crosslinks (93) (Figure 2*a*). Coa, by contrast, has the unique ability to associate with a molar excess of fibrinogen via its C-terminal repeat (R) domain, comprising up to twelve tandem repeats of a conserved 27-amino-acid peptide (94). This interaction allows Coa to localize at the cell surface of *S. aureus* by binding to fibrinogen retained by cell wall anchored proteins such as ClfA and favors formation of a staphylothrombin-generated fibrin shield in the immediate vicinity of the staphylococci (L. Thomer, unpublished). These findings explain earlier observations of cell-bound (Coa) and cell-free coagulases (vWbp) (95).

The hemostatic system is responsible for preventing the dissemination of microbial invaders (96). Microbe-specific activation of the coagulation cascade is mediated by the intrinsic pathway, also known as contact system, and is activated by negatively charged structures present on the surface of bacteria (44) (Figure 2*a*). Such structures are recognized by factor XII, which subsequently auto-activates into factor XIIa (97). Factor XIIa, in turn, converts prekallikrein and factor XI into their active proteolytic forms, kallikrein and FXIa, respectively (97). FXIa converges with the extrinsic coagulation cascade and ultimately

leads to fibrin clot formation, while activated plasma kallikrein cleaves the nonenzymatic cofactor high-molecular-weight kininogen and releases the proinflammatory peptide bradykinin (97). As recently reviewed by Berends et al. (44), the antibacterial effects of coagulation are manifold; first and foremost, bacteria become immobilized inside a fibrin meshwork, preventing their spread into surrounding tissues. Bradykinin, by contrast, has direct antimicrobial activity, functions as a chemoattractant, and induces vascular leakage, thereby recruiting additional immune cells (44). Peptides derived from other coagulation factors, such as thrombin or fibrinogen, have also been shown to be antibacterial, whereas fibrinopeptides A and B are potent chemoattractants (44). The relevance of this innate defense mechanism has been demonstrated for *Streptococcus pyogenes*, the causative agent of human pharyngitis and skin infections (98, 99).

Whereas the activation of the coagulation cascade represents a universal host defense against bacterial infections, *S. aureus* has evolved unique virulence strategies that usurp the hemostatic system for pathogen survival and replication in infected tissues. By circumventing both branches of the physiological blood coagulation cascade and activating prothrombin in a nonproteolytic manner that restricts the substrate specificity of the resulting staphylothrombin complex to fibrinogen, *S. aureus* avoids the vast majority of proinflammatory signaling described above. In fact, the bacterium secretes SSL10 to inhibit host blood coagulation by binding to the γ-carboxyglutamic acid domain of prothrombin and factor Xa (100) (Figure 2*a*). SSL10 only weakly interferes with staphylothrombin activity, indicating that its main function is to dampen a potential activation of the physiological coagulation cascade via the contact pathway while allowing staphylococcal coagulation to occur (100).

STAPHYLOCOCCAL INTERFERENCE WITH FIBRINOLYSIS

Fibrinolysis is the reversal of coagulation, as fibrin clots are being degraded by proteases and injured tissues are being repaired (101). A key factor for the initiation of fibrinolysis is plasminogen, a plasma zymogen. During repair, plasminogen is converted to enzymatically active plasmin by host plasminogen activators, urokinase or tissue type plasminogen activator (101). Similar to the coagulation cascade, fibrinolysis can be viewed as a cascade of protease activation and inhibitory events that are integrated with coagulation and inflammation as well as wound healing and angiogenesis. *S. aureus* secretes Sak, which binds plasmin and modifies its active site for catalytic activity (102). The Sak-plasmin complex is highly fibrin-specific and subject to rapid inhibition by the endogenous plasmin inhibitor α-2-antiplasmin unless it is bound to fibrin (103). Sak·plasminogen activity is therefore restricted to areas of fibrin deposition. In contrast to *coa*, *vwb* and *clfA*, which belong to the core genome of *S. aureus*, *sak*, the structural gene for Sak, is located in the immune evasion cluster of *hlb*-converting phages along with *scn* and *chp* (104).

Interestingly, *sak* expression is inversely correlated with a lethal outcome of *S. aureus* bacteremia, as *sak*-deficient isolates are four times more likely to cause death among patients (105). The high frequency of Sak production in nasal isolates suggests that Sak expression may be an adaptive mechanism of *S. aureus* colonization of its human host (105). Analyses on the contributions of *sak* toward *S. aureus* colonization and pathogenesis are

confounded by the fact that Sak is selective for human plasminogen but inactive for plasminogen from many other vertebrates (105, 106). When the contribution of Sak to systemic infection was interrogated with transgenic mice expressing mouse and human plasminogen, *sak* expressing *S. aureus* strains displayed reduced virulence as evidenced by reduced mortality of infected animals and lower bacterial burden in infected organ tissues (107).

Given the importance of staphylothrombin-mediated fibrin deposition for bloodstream infection and formation of abscess lesions in organ tissues (*vide infra*) and the seemingly contradictory activities of pro- and anticoagulant virulence factors, Sak may play a role in disease manifestations such as SSTIs rather than bloodstream infections. In support of this hypothesis, plasminogen activation by Sak was shown to enhance local spreading of *S. aureus* in a mouse skin infection model (108). Of note, despite enhanced virulence in the skin, Sak expression did not lead to systemic dissemination of bacteria, suggesting that the virulence mechanism of Sak may be restricted to the dermal microenvironment (108).

STAPHYLOCOCCAL EXIT FROM THE BLOODSTREAM

In order to disseminate to organ tissues and seed abscess lesions, *S. aureus* needs to leave the vasculature. Most adhesion factors identified to date are members of the MSCRAMM family and rely on bridging molecules present in plasma or released by endothelial cells for their adhesive properties (109). Most prominent in this context are FnBPA and FnBPB, which were shown to significantly contribute to adherence of *S. aureus* to endothelial cells in vitro and in vivo (110, 111) (Figure 1*e*). Their role in escape from the bloodstream is particularly intriguing because they have also been implicated in staphylococcal invasion of host cells. The fibronectin-binding repeats of FnBPs engage multiple fibronectin molecules, which, in turn, are recognized by the $\alpha_5\beta_1$ integrin on the endothelial surface to trigger bacterial uptake (112). Although it is unclear how staphylococci exit endothelial cells on their basal face, the contribution of FnBPA-mediated invasion to bloodstream infection was demonstrated in a murine model (113). Of note, staphylococcal strains expressing *fnbA* alleles with fewer fibronectin-binding repeats were observed to be defective in endothelial cell invasion and subsequent systemic spread, yet these strains promoted *S. aureus* survival in blood as well as immune signaling (113).

Teichoic acids, anionic glycopolymers found in the cell envelope of many different Gram-positive bacteria, have been implicated as nonproteinaceous adhesion and endothelial invasion factors. WTA was found to contribute to *S. aureus* attachment to endothelial cells in vitro, leading to reduced dissemination into renal tissues in a rabbit model of infective endocarditis (61). LTA, on the other hand, despite not contributing to adhesion to endothelial cells, affected invasion of endothelial cells and dissemination into brain tissue (114). The receptors for WTA or LTA on endothelial cells and the molecular mechanisms for uptake of *S. aureus* into these cells are, however, not known (Figure 1*e*).

vWF, a large glycoprotein stored in Weibel-Palade bodies of endothelial cells, is released in multimeric form into the bloodstream following vascular injury. vWF binds FVIII, collagen and platelets, forming a complex with platelet glycoprotein to quench blood loss, effectively

coordinating platelet deposition and fibrin formation at sites of vascular injury (115). vWF has been implicated in *S. aureus* adhesion to vascular endothelia under shear stress (116), and secreted vWbp has been implicated as a bridging molecule for this event (117). vWbp is secreted into the extracellular milieu and not associated with bacterial surfaces (9); thus, it is not clear how vWbp and vWF may promote *S. aureus* adhesion to the vasculature. Alternatively, vWbp·prothrombin derived fibrin fibers may promote endothelial adherence by inducing microthrombi with staphylococci embedded with fibrin shields (117). This model highlights a possible route for *S. aureus* to invade organ tissues: blocking blood flow in the microvasculature via staphylococcal agglutination (Figure 1*e*). This mechanistic model may be correlated with the clinical phenomenon of disseminated intravascular coagulation, as occurs during *S. aureus* sepsis in humans and in a pig model for bloodstream infection (118).

Another important route for staphylococcal exit from the vasculature may be the disruption of endothelial barriers. In addition to its pore-forming activity, Hla binds to the receptor, ADAM10, and upregulates its metalloprotease activity (119). By cleaving vascular endothelial cadherin, which engages in homotypic intercellular interactions, ADAM10 compromises endothelial barrier function and promotes vascular leakage (119) (Figure 1*e*). Although this has not yet been experimentally demonstrated, *S. aureus* may exploit the increased vascular permeability for entry into host tissues.

As reported for several other bacterial pathogens, *S. aureus* may rely on professional phagocytes for extravasation and dissemination (Figure 1*e*). Considering its arsenal of immune evasion factors providing for intracellular survival, *S. aureus* can reside in the intracellular vacuoles of monocytes or macrophages for several days or enter the cytoplasm of immune cells (120). Intracellular survival was shown to require several virulence factors, including SpA, SrtA, and aureolysin, and may provide opportunities for staphylococcal travels from the bloodstream into organ tissues (120). To study the possibility that phagocytes may act as Trojan horses for the travels of staphylococci, mouse neutrophils were isolated from the peritoneal cavity of mice infected with *S. aureus* and transferred into naïve mice: infected neutrophils were indeed sufficient to establish abscess lesion (121).

STAPHYLOCOCCAL ABSCESS FORMATION AND PATHOGENESIS

S. aureus is endowed with several mechanisms to resist and escape killing in the bloodstream of infected hosts, and the pathological product of *S. aureus* invasion is the formation of deep-seated abscess lesions. Herein simply referred to as abscesses, these lesions differ from superficial skin lesions (17). In principle, abscess formation is considered a default response to biological (infectious), chemical and physical trauma, whereby damaged tissues are encapsulated, liquefied, drained and eventually repaired. A notable feature of staphylococcal abscess lesions is the massive infiltration of immune cells, responding to cellular destruction and proinflammatory signals, and resulting in the liquefaction of tissue into purulent exudates that are eventually drained. Unlike *S. aureus*, however, entry of *S. epidermidis* or other nonpathogenic or opportunistic pathogens does not elicit abscess lesions and purulent exudate in humans or experimental animals. Further, mutations in virulence genes diminish the ability of *S. aureus* to establish purulent abscess

lesions in experimental animals (122). When studied in mice, histopathological analyses of *S. aureus* lesions in renal tissues revealed a pathogen-driven, developmental process, supporting staphylococcal replication and dissemination (122).

Earlier work described four stages of staphylococcal abscesses formation (123). Following entry into the bloodstream, *S. aureus* blocks innate defenses of its host and enters organ tissues. During this first stage (stage I), a characteristic drop in the staphylococcal load is observed in the bloodstream within hours of inoculation and this drop is accompanied by an increased burden of *S. aureus* in many different organ tissues (122). Staphylococcal invasion of host tissues, in turn, triggers tissue infiltration with large numbers of immune cells, predominantly neutrophils and macrophages (stage II). As early as two days post infection, these immune cell infiltrates are readily visualized in hematoxylin-eosin stained thin-sectioned tissue (122). During stage II, staphylococci in infected tissues display no discernable organization and appear to reside mostly within neutrophils (122). Within the next 48–72 h, the morphological features of *S. aureus* abscesses are profoundly altered, with staphylococcal abscess communities at the center of the lesion, enclosed by fibrin deposits, and surrounded by concentric layers of immune cells (Figure 3). Eosinophilic deposits, likely made up of fibrin, demarcate stage III lesions from healthy, uninfected tissues. During stage III, immune cell infiltrates are initially made up of neutrophils, arranged in concentric layers of apoptotic, healthy, and apoptotic appearance with other immune cells, for example macrophages and lymphocytes, at the peripheries of lesions (66, 122) (Figure 3*a*). Staphylococcal abscesses display multiple morphological changes over time. By day 15 post infection, SACs have expanded; most immune cells appear necrotic, with cellular detritus accumulating; and foam cells (macrophages) are positioned at the periphery (Figure 3*b*). Eventually, T and B lymphocytes as well as macrophages infiltrate lesions, which are slowly pushed toward organ surfaces and eventually rupture, releasing purulent exudate and staphylococci for renewed entry into the bloodstream or dissemination to new hosts (stage IV) (122).

S. aureus lacking sortase A (Δ*srtA*) cannot establish abscess lesions following bloodstream infection of mice, and the Δ*srtA* mutant cannot persist in infected tissues over a period of 15 days (122). *S. aureus* mutants with defects in individual surface protein genes retain their ability to generate abscess lesions, indicating that the Δ*srtA* phenotype is caused by the cumulative defect for all sortase-anchored surface proteins. Nonetheless, lesions derived from variants with mutations in *clfA*, *clfB*, *isdC* or *sdrC* were characterized by reduced staphylococcal load in infected tissues, whereas *sdrD*, *isdA*, *isdB* or *spa* mutants exhibited reduced numbers of abscess lesions (122) (Table 1). These data suggest that the heme-scavenging proteins IsdA, IsdB, and IsdC promote staphylococcal replication in infected tissues, likely providing iron from host hemoglobin and haptoglobin to support the nutritional requirements of *S. aureus*. The role of SdrD in the pathogenesis of *S. aureus* bloodstream infection and abscess formation is not known. A member of the MSCRAMM and SD repeat families of surface proteins, SdrD mediates staphylococcal adherence to desquamated epithelial cells (124). It seems plausible that SdrD may exert additional ligand activities supporting *S. aureus* abscess formation during stages II–III (Table 1). SpA binds Igs via their Fcγ domain, which inhibits opsonization and phagocytosis of bacteria (125). In fact, *S. aureus* expressing *spa$_{KK}$*, a SpA variant that no longer binds to the Fcγ domain of

Igs, is more readily phagocytosed and killed in the bloodstream of infected mice resulting in reduced numbers of abscess lesions (125). SpA B cell superantigen activity and escape from humoral immune responses is based on its ability to crosslink the Fab heavy chains of V_H3 clan B cell receptors (126); this activity is, however, not required for *S. aureus* abscess formation in mice (125).

Secreted Coa is found in the immediate proximity of staphylococcal abscess communities and their surrounding fibrin deposits, whereas vWbp is localized throughout and in the periphery of abscess lesions (77) (Table 1). In an effort to dissect the relative contribution of Coa and vWbp to fibrin deposition even further, staphylococcal abscess formation was reconstituted in a three-dimensional collagen matrix supplemented with host fibrinogen and prothrombin (55). Formation of two concentric fibrin structures was observed, with Coa being involved in formation of the inner fibrin capsule and vWbp giving rise to an outer fibrin meshwork (55). Using deletion mutants, both fibrin layers were shown to protect the central staphylococcal community against attacking neutrophils (55). In addition to helping staphylococci escape phagocytic clearance in the bloodstream, the two coagulases, likely in tandem with ClfA, also contribute to abscess development by concerting the formation of the distinct layers described above.

Immune cells, in particular neutrophils, are an integral part of abscess lesions. This begs the question of how staphylococci strike a balance between blocking their own clearance from these phagocytes and recruiting cells to develop abscess lesions for their dissemination to new sites and or new hosts. It seems unlikely that the dramatic infiltration of immune cells at stage II of abscess formation can be explained by massive bacterial replication because colonies of staphylococci are not discernable early during infection (122). Instead, *S. aureus* seems to actively recruit immune cells through the release of proinflammatory molecules perceived by innate immune sensors such as TLRs. This conjecture is supported by the observation that *S. aureus* mutants lacking lipoprotein diacyl-glyceride transferase (Lgt), which catalyzes diacyl-glycerol modification of lipoprotein precursors, are defective in the recruitment of immune cells (127). Although *lgt* mutants can replicate in host tissues, mutant colonies are not surrounded by neutrophils and do not form typical abscess lesions (127). More recently, it was found that the clonal complex 30 MRSA lineage, which is associated with HA bacteremia and high occurrence of metastatic abscesses, expresses an allelic variant of PSMα3 that shows reduced cytolytic and chemotactic potential toward human neutrophils (128). In a mouse model of renal abscess formation, infection with this PSMα3 allele was correlated with increased bacterial load and increased numbers of infectious lesions in renal tissues (128) (Table 1). As described above, PSMs are recognized by formyl peptide receptors to elicit proinflammatory signals for the recruitment of immune cells to staphylococcal abscess lesions (128). Whereas increased recruitment of immune cells may precipitate staphylococcal elimination, diminished recruitment, for example due to secretion of an altered PSM, may provide staphylococci with the means for escape from innate defenses and persistence in host tissues. Although not tested experimentally, the products of SSL5, SSL10, CHIPS, and FLIPr, by impeding chemotaxis of neutrophils, may also contribute to the maturation and pathology of abscess lesions (Table 1).

PERSPECTIVES

Vaccines that protect humans against bacterial pathogens elicit antibody responses to either neutralize a key virulence factor, for example *Corynebacterium diphtheriae* diphtheria toxin (129), or induce opsonophagocytic killing of the invading pathogen, typically by binding to bacterial capsular polysaccharides (130, 131). The latter are currently used to establish protective immunity against *Neisseria meningitidis*, *Haemophilus influenzae* and *Streptococcus pneumonia* (132). Applying similar approaches to *S. aureus* vaccine development has thus far not proved successful, as neither antibodies against staphylococcal polysaccharide (133) nor antibodies against secreted virulence factors could protect humans against staphylococcal disease (134, 135). Such failures can be explained by the multifactorial, parallel strategies that *S. aureus* deploys toward disease establishment and by the wide spectrum of the pathogen's immune evasive attributes that block opsonophagocytic killing. In other words, *S. aureus* secretes so many virulence factors that formylation of subunit vaccines would have to involve dozens of antigens and does not seem feasible. Further, opsonophagocytosis of staphylococci is blocked by the microbe's agglutination pathway, by complement evasion factors and by staphylococcal protein A, each presenting formidable obstacles toward antibody-induced clearance of *S. aureus*. Thus, the development of *S. aureus* vaccines is indeed a daunting task. Recent work has explored new vaccine strategies by targeting the immune-evasive attributes of the pathogen (136–138). Although it is not possible to predict success for such endeavors, the approaches are certainly innovative and may stimulate parallel work on vaccines for other pathogens with expertise at evading human immune responses, including *Mycobacterium tuberculosis*.

Acknowledgments

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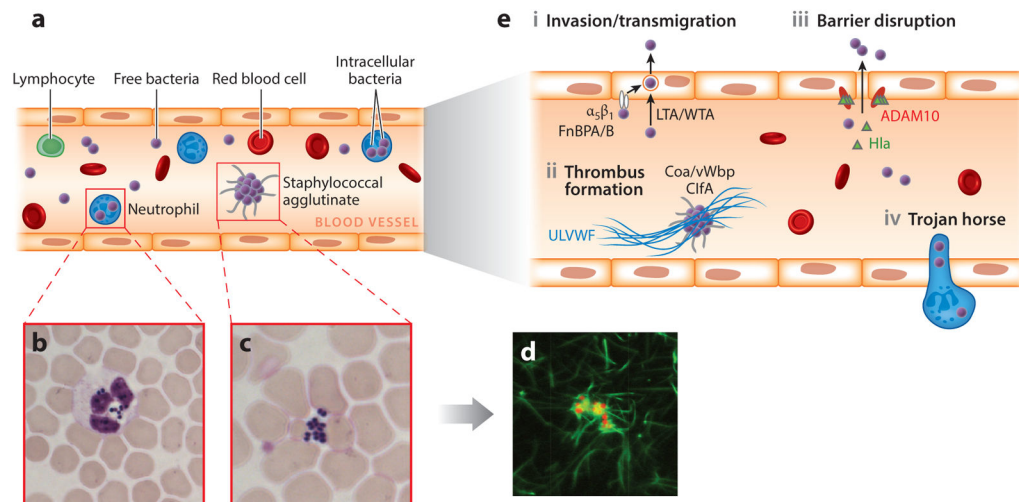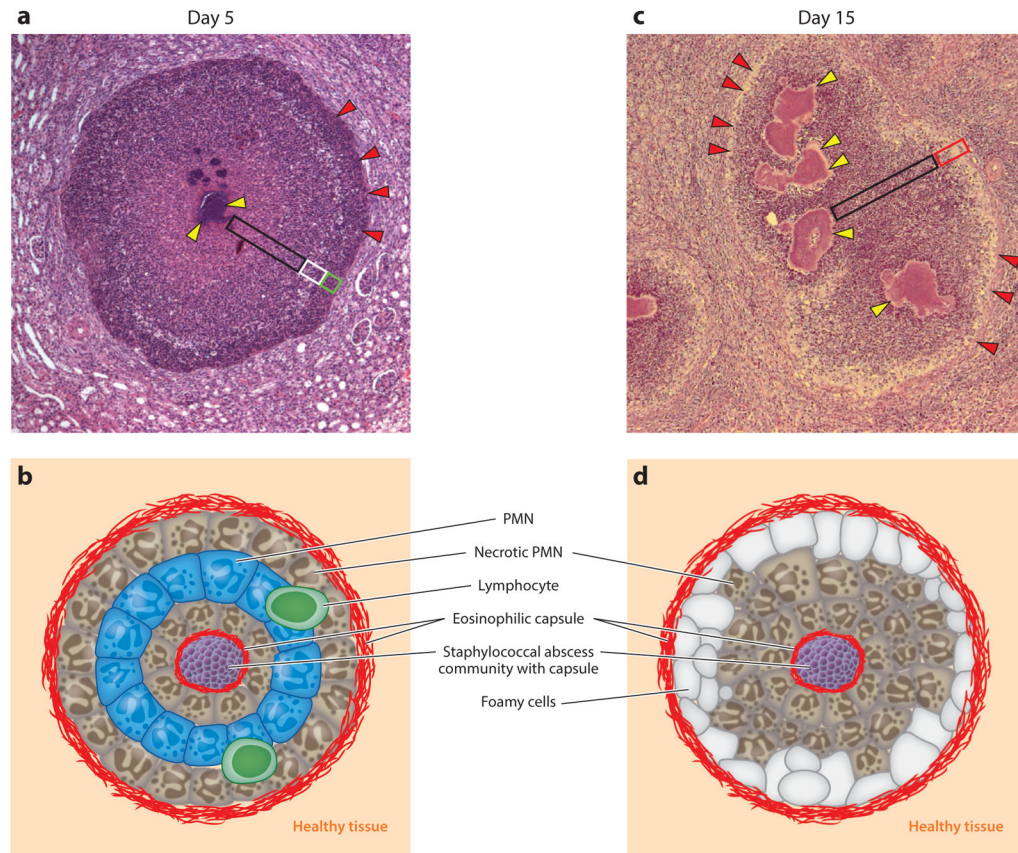
Figure 1.

Survival of *Staphylococcus aureus* in the bloodstream and dissemination into host tissues. (*a*) Following intravenous inoculation of mice with ~10^7 colony-forming units (CFU), *S. aureus* survives in the bloodstream and disseminates from the vasculature into organ tissues. Bacteria from the initial inoculum cannot be detected in the bloodstream within 24 h. (*b–c*) Microscopy of Giemsa-stained blood samples from mice infected for 2 h reveals staphylococci that are (*b*) associated with neutrophils or (*c*) agglutinated outside of immune cells. Staphylococci phagocytosed by neutrophils deploy several virulence strategies that ensure their survival (see text for details). (*d*) Extracellular *S. aureus* cocci expressing mCherry (*red fluorescence*) interact with fibrin (*green fluorescence*) to generate large bacterial agglutinates covered with a shield of fibrin cables, thereby preventing phagocytosis. The image shows *S. aureus* mixed for 5 min with anticoagulated human plasma that has been supplemented with 5% Alexa488-conjugated human fibrinogen. Staphylococcal agglutination requires Coa/vWbp (coagulases) and prothrombin as well as ClfA (agglutinin) and fibrinogen. Agglutinins are *S. aureus* surface proteins that bind coagulase-derived fibrin cables to produce large aggregates of bacteria covered with fibrin. (*e*) Models for *S. aureus* exit from the bloodstream. (*i*) FnBPA and FnBPB (staphylococcal surface proteins) bind fibronectin and interact with integrin $\alpha_5\beta_1$ on the surface of the vascular endothelium, thereby triggering invasion of cells and transmigration. Wall teichoic acid (WTA) and lipoteichoic acid (LTA) of *S. aureus*, polymers in the bacterial envelope, have also been proposed to promote staphylococcal invasion of host cells. (*ii*) Staphylococci induce formation of fibrin thrombi via Coa/vWbp- and ClfA-mediated agglutination and bind to von Willebrand factor (vWF) on endothelial surfaces, generating Ultra Large vWF (ULVWF) polymers. (*iii*) Staphylococci secrete Hla, a toxin that binds to the ADAM10 receptor to disrupt the physiological barrier functions of the vascular endothelium. (*iv*) The Trojan horse model, whereby neutrophils with intracellular *S. aureus* extravasate to deliver bacteria into host tissues.
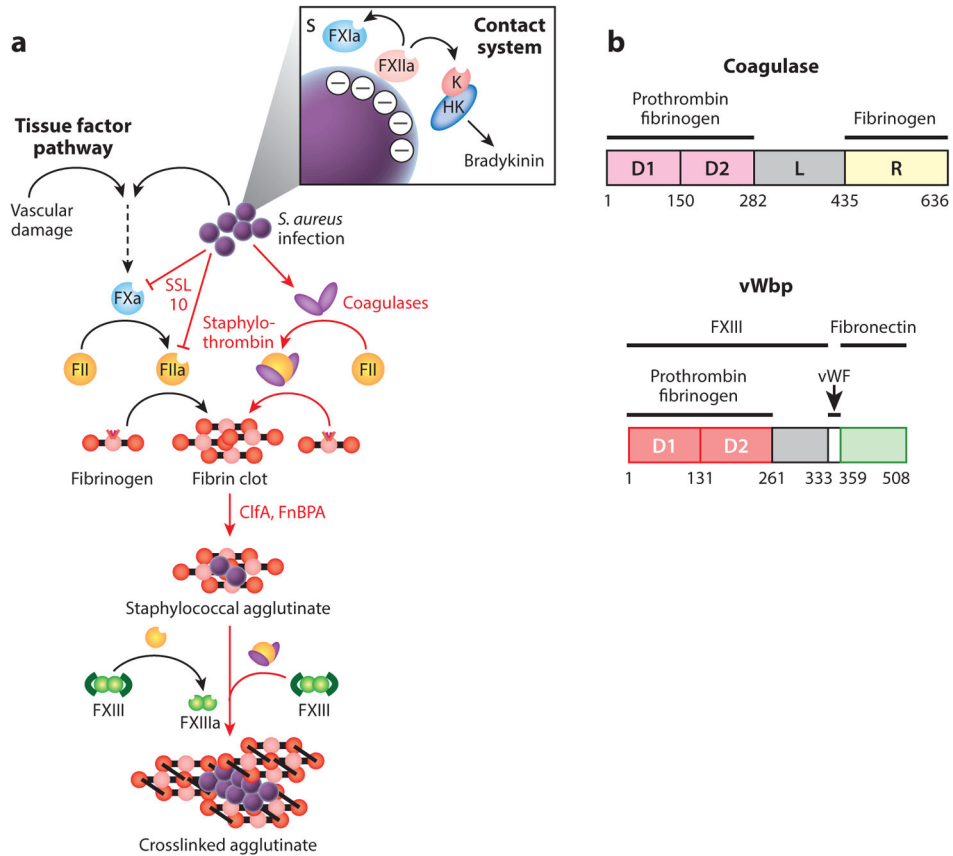
Figure 2.

Agglutination and *Staphylococcus aureus* interference with host hemostasis. (*a*) Vascular damage and tissue factor or contact activation and bradykinin are pathways for hemostasis—a cascade of proteolytic activation for the zymogens of clotting factors culminating in the conversion of prothrombin (FII) to thrombin (FIIa), which cleaves fibrinopeptides A and B from fibrinogen, thereby triggering self-assembly of fibrin cables either to stem blood loss from injured vasculature or to immobilize pathogens that have activated the contact system. *S. aureus* bypasses the host hemostasis pathways to activate FII/prothrombin via the secretion of coagulases (Coa and vWbp), thus yielding staphylothrombin and, via nonproteolytic activation of fibrin-stabilizing factor (FXIII), the transglutaminase responsible for crosslinking fibrin cables. ClfA, an agglutinin of the surface of *S. aureus*, captures staphylothrombin-derived fibrin cables, thereby agglutinating staphylococci into large aggregates of bacteria enclosed by a fibrin shield. (*b*) A diagram illustrating the primary translational products of Coa and vWbp from *S. aureus* strain Newman; the catalytic D1 and D2 domains are shown in light and dark red boxes, and the R domain of coagulase with repeats is shown in yellow. The function of the linker domain (L) is not known. The number of repeats in the R region of coagulase varies between strains. The binding sites for prothrombin, fibrinogen, FXIII, and fibronectin are delineated. Abbreviation: vWF, von Willebrand factor.

Figure 3.

Staphylococcus aureus abscess lesions in murine renal tissue. (*a*) Microscopy image of hematoxylin-eosin-stained, thin-sectioned renal tissue 5 days following intravenous inoculation with *S. aureus*. Bacteria are organized into staphylococcal abscess communities (SACs) (*yellow arrowheads*) at the center of the lesion. Depending on sectioning, SACs may appear fragmented; however, bacterial communities remain connected and are enclosed by eosinophilic deposits of fibrin and surrounded by zones of necrotic neutrophils (*black box*), healthy-appearing neutrophils (*white box*), and necrotic immune cells (*green box*). Abscess lesions are demarcated from renal tissues by another layer of eosinophilic deposits (*red arrowheads*), through which immune cells enter the lesion. (*b*) Schematic illustrating the histopathology features of a 5-day-old *S. aureus* abscess lesion. (*c*) Microscopy image of hematoxylin-eosin-stained, thin-sectioned renal tissue 15 days following intravenous inoculation with *S. aureus*. The 15-day-old lesion differs from the 5-day-old lesion in that SACs have expanded and the layering of healthy and necrotic immune cells has been supplanted by diffuse infiltrates of immune cells, cellular detritus, liquefaction (*black box*), and foam cells positioned at the periphery of the lesion (*red box*). SACs remain enclosed by fibrin deposits (*yellow arrowheads*). An outer eosinophilic layer (*red arrowheads*) demarcates the lesion from large numbers of immune cells and from the cellular detritus that has replaced renal tissue architecture in the vicinity of the abscess lesion. (*d*) Schematic

illustrating the histopathology features of a 15-day-old *S. aureus* abscess lesion. Abbreviation: PMN, polymorphonuclear leukocyte.

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Table 1

Secreted virulence factors and their possible contributions to abscess formation and pathology

Name	Gene	Proposed function[a]	Virulence defect associated with loss of gene[b]
Secreted virulence factors involved in survival in the bloodstream			
Adenosine synthase	*adsA*	Synthesis of immune suppressors Ado, dAdo	Decreased survival in blood
Nuclease	*nuc*	Degradation of NETs	Decreased survival in blood
Clumping factor A	*clfA*	Agglutination	Increased phagocytic uptake
Coagulases	*coa* *vwb*	Agglutination	Increased phagocytic uptake
Protein A (SpA)	*spa*	Binding of the Fcγ domain of Igs	Increased opsonization and phagocytosis of bacteria
Leukocidins	*hlgAB* *hlgCB* *lukAB*	Lysis of leukocytes	Unknown Unknown Decreased survival in blood
Fibronectin-binding repeat protein A, B	*fnBPA* *fnBPB*	Recognition of fibronectin-FnBR complexes by $\alpha_5\beta_1$ integrin; fibrinogen binding	Unknown
Staphylococcal complement inhibitor (SCIN), SCIN-B, SCIN-C	*scin* *scinB* *scinC*	Blockage of C3 convertases	Unknown
Extracellular complement–binding protein	*ecb*	Blockage of C3 convertases	Unknown
Aureolysin	*aur*	Degradation of antimicrobial peptides	Unknown
Secreted virulence factors required to breach the bloodstream			
α-Hemolysin	*hla*	Disruption of epithelial or endothelial surfaces	Reduced bacterial loads in organs
Serine Aspartic Repeat protein C, D	*sdrC* *sdrD*	Members of the MSCRAMM, ligands unknown	Reduced bacterial loads in organs
Secreted factors required for bacterial replication and persistence in abscesses			
Iron surface determinant A, B, C	*isdA* *isdB* *isdC*	Heme iron scavenging	Reduced bacterial loads
Coagulases	*coa* *vwb*	Coagulation, agglutination	Reduced bacterial loads and numbers of abscesses
Protein A (SpA)	*spa*	B cell superantigen activity that prevents humoral immune response	Reduced bacterial loads and numbers of abscesses in organs
Lipoprotein diacyl-glyceride transferase	*lgt*	Lipidation of lipoproteins	Hypervirulence, abscesses devoid of immune cells due to loss of TLR2 ligands
Extracellular adherence protein	*eap*	Blockade of neutrophil adherence to endothelia	Reduced bacterial loads in organs
Extracellular fibrinogen–binding protein	*efb*	Blockade of C3 convertase; fibrinogen binding	Reduced bacterial loads in organs
Phenol soluble modulins α3	*psmα3*	Generation of inflammatory signals for the recruitment of immune cells	Increased bacterial loads in organs
Staphylococcal superantigen-like 5, 10	*ssl5* *ssl10*	Impediment of neutrophil chemotaxis	Unknown
Chemotaxis inhibitory protein	*chips*	Impediment of neutrophil chemotaxis	Unknown

Secreted virulence factors involved in survival in the bloodstream			
Name	Gene	Proposed function[a]	Virulence defect associated with loss of gene[b]
Formyl peptide receptor-like inhibitory proteins	*flipr* *flipr-like*	Impediment of neutrophil chemotaxis	Unknown

[a]The proposed function is derived from in vitro biochemical experimentation.

[b]Virulence defect was tested as survival of bacteria in whole blood or enumeration of bacterial loads in animal organ tissues following infection.