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A play in four acts: *Staphylococcus aureus* abscess formation

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Abstract

Staphylococcus aureus is an important human pathogen that causes skin and soft tissue abscesses. Abscess formation is not unique to staphylococcal infection and purulent discharge has been widely considered a physiological feature of healing and tissue repair. Here we present a different view, whereby *S. aureus* deploys specific virulence factors to promote abscess lesions that are distinctive for this pathogen. In support of this model, only live *S. aureus* are able to form abscesses, requiring genes that act at one or more of four discrete stages during the development of these infectious lesions. Protein A and coagulases are distinctive virulence attributes for S. *aureus*, and humoral immune responses specific for these polypeptides provide protection against abscess formation in animal models of staphylococcal disease.

Staphylococci and *Staphylococcus aureus*

Sir Alexander Ogston observed ‘micrococci’ in pus from surgical wound infections and named the bacterium *Staphylococcus*. Using eggs to isolate pure cultures for the inoculation of rabbits, he demonstrated that staphylococci cause the development of abscesses in infected animals. During the Ninth Surgical Congress in Berlin (1880), Ogston reported his results, firmly establishing staphylococci as the etiological agent of suppurative abscesses. To date, 36 species and 18 subspecies of the genus *Staphylococcus* have been distinguished[1, 2]. Ogston’s microbe, *Staphylococcus aureus*, remains the most prominent member among this group, as it is the most frequent cause of human skin or soft tissue abscesses[3]. In addition to abscesses, *S. aureus* strains can cause pneumonia, toxic-shock syndrome, exfoliative skin disease, and enteritis[3]. Several unique biological traits are associated with *S. aureus*, including the ability to bind to immunoglobulin, agglutinate and coagulate blood or plasma (Figure 1)[3].

S. aureus and several other staphylococcal species colonize the human skin, nails, and nares and disseminate among recipient host populations via physical contact and aerosols[3]. The massive consumption of antibiotics over the past 50 years has led to the selection of drug-resistant strains, designated MRSA for methicillin-resistant *S. aureus*[4]. Although no longer in clinical use in the United States, methicillin was initially used for the treatment of penicillin-resistant strains; penicillinase is unable to cleave methicillin. MRSA isolates are now widespread[5]. The basis for their resistance is a penicillin-binding protein that cannot be inhibited by methicillin or oxacillin. As a result, vancomycin has been used as the last-resort

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Conflict of Interest
Alice Cheng, Andrea DeDent, Olaf Schneewind and Dominique Missiakas are named inventors on a patent owned by The University of Chicago, which is the subject of a license agreement with Novartis Vaccines and Diagnostics.

antibiotic for MRSA infections. However, *S. aureus* isolates that have vancomycin-resistant (VRSA) or vancomycin-intermediate (VISA) phenotypes have emerged by generating altered substrates for cell wall synthesis and no longer associate with vancomycin or related glycopeptide drugs[6–8]. MRSA are a frequent cause of hospital-acquired infection in the United States[9]. Over the past decade, community-acquired infections (CA-MRSA) increased in abundance and severity[9]. One such clone has swept over the United States and currently colonizes a large segment of the population[10]. An FDA approved vaccine that can prevent *S. aureus* infections caused by MSSA (methicillin-sensitive *S. aureus*), MRSA, CA-MRSA, VISA or VRSA strains is not yet available.

Breaches in local defense, for example skin cuts or hair follicle trauma, provide opportunities for staphylococcal invasion, which typically manifests as abscess formation: the accumulation of pus accompanied by severe inflammation of surrounding tissues[3]. Staphylococci in pus disseminate onto skin surfaces or enter circulating lymph and blood, which leads to the formation of abscesses at new sites[3]. *S. aureus* is a frequent cause of sepsis, often the consequence of invasive disease originating from one or more abscesses. About one-fifth of staphylococcal skin and soft tissue infections that improve under antibiotic therapy flare up again at a later time[11]. This phenomenon, as well as clinical data on *S. aureus* infected individuals, suggests that some humans do not acquire protective immunity following infection[3]. Here we review what is known about *S. aureus* abscess formation and the molecular mechanisms that enable the pathogen to form these lesions and suppress the immune responses of its host.

Abscess formation in stages

Staphylococci that disseminate through the bloodstream enter peripheral tissues and seed infectious lesions, initially inducing inflammatory responses that attract neutrophils, macrophages, and other phagocytes[3]. The responsive invasion of immune cells to the site of infection is accompanied by liquefaction necrosis and the release of pus with replicating staphylococci into circulating body fluids[12–14]. Defensive host responses to staphylococcal invasion include fibrin deposits to shield healthy tissues from the disseminating microbe. Upon abscess drainage or surgical excision of infected lesions, lost organ tissues can be replaced with fibrotic scars. However, these events cannot be considered specific host responses to *S. aureus* invasion; they are induced even with chemical trauma, physical insult or injection of sterile biological materials into organ tissues[15].

We suggest that abscess formation and staphylococcal entry into purulent material is a pathogen driven process that usurps the default responses of its infected host to enhance microbial replication and dissemination. In support of this hypothesis, several pathogen-specific genes are required for the ability of *S. aureus* to establish abscess lesions (*vide infra*)[14, 16]. Following entry into organ tissues, staphylococci attract immune cells and replicate in their presence to eventually replace physiological epithelia with distinctive lesions[14]. Within four days of infection, the pathogen has organized itself as a staphylococcal abscess community (SAC) at the center of the lesion, shielded from host immune cells by a surrounding pseudocapsule comprised of fibrin deposits[16]. These lesions grow through the arrival of immune cells and the destruction of healthy tissues associated with neutrophil necrosis. Staphylococci disseminate from these lesions with the draining pus into circulating body fluids.

Using C57BL/6 and BALB/c mice in an intravenous challenge model, infected animals produced abscess lesions in all tissues examined similar to those observed in humans[14]. We liken the pathogenesis of staphylococcal disease to a play that is staged in four acts (Figure 2). Following intravenous injection, *S. aureus* survives in the bloodstream as an extracellular

pathogen or when phagocytosed by neutrophils (Stage I). Upon distribution of staphylococci to peripheral tissues, neutrophils as well as other immune cells are recruited to the site of infection (Stage II). *S. aureus* reprograms infected lesions to replicate as a SAC at their center, separated from surrounding immune cells by a pseudocapsule with fibrin deposits (Stage III). Movement of lesions to organ capsules and their subsequent rupture releases *S. aureus* with purulent exsudate to other sites, where the pathogen repeats this infectious program (Stage IV). Without antimicrobial therapy, staphylococci cannot be cleared by host immune responses from abscess lesions and infected animals typically succumb to disease within 30–60 days[14].

Protagonists of the plot

Stage I: survival of staphylococci in blood

Following intravenous inoculation of mice with a sublethal dose of bacteria (~10^7 colony forming units, CFU), 99.9% of the original inoculum disappears from the vasculature within 6 h (Figure 3a). Dissemination to organ tissue occurs within 3 h followed by rapid decline (3–12 h) and measurable replication after 24 h (Figure 3a)[14]. Unless staphylococci survive or replicate in blood and circulating lymph fluids, this pathogen could not disseminate to new sites within an infected host. Thus, the first stage of the plot is to describe the actors in the play that lead to disease crisis and dramatic endings of *S. aureus* infections. To survive in blood, *S. aureus* must escape a variety of innate immune mechanisms, including antimicrobial peptides, complement, and phagocytic killing[17, 18]. The principal defense against *S. aureus* is provided by neutrophilic polymorphonuclear leukocytes, which comprise 60–70% of human white blood cells[19]. Reactive oxygen species, along with the acidic vacuolar environment, prove toxic to many microbes, and help curtail bacterial spread. *S. aureus* produces the carotenoid staphyloxanthin[20, 21], a membrane-bound pigment that scavenges reactive oxygen species and protects *S. aureus* from neutrophil killing[22]. Further, staphylococci deploy a group of secreted peptides, termed phenol-soluble modulins (PSMs), to target human neutrophils for destruction by disrupting the integrity of their plasma membrane[23].

The complement cascade plays a central role in innate host defenses to *S. aureus*[24–26]. By binding to microbial surfaces, C3 and C5 convertases deposit complement and mediate phagocytic killing of bacteria. The associated release of C3 and C5 fragments further serves as a chemoattractant for phagocytes. *S. aureus* subverts these mechanisms by secreting factors inhibitory for complement activation and neutrophil chemotaxis[27, 28]. Although not part of the core *S. aureus* genome, these prophage-encoded factors are expressed by many virulent strains[29].

In addition to secreted factors, staphylococci rely on cell wall-anchored surface proteins for their survival in blood[30]. Clumping factor (ClfA), a sortase-anchored product whose N-terminal domain is displayed on the bacterial surface, binds fibrinogen[31]. This biochemical attribute triggers staphylococcal agglutination in the presence of fibrinogen or fibrin, i.e. in lymph fluids or blood[31]. How agglutination ensures staphylococcal survival is not entirely clear. On one hand, the extremely large size of agglutinated staphylococci can be thought to interfere with phagocytosis[32, 33]. On the other, ClfA enables staphylococci to adhere to fibrin deposits on the vascular endothelium, providing a niche for pathogen replication at the periphery of circulating fluids. The latter mechanism is certainly essential for the pathogenesis of endocarditis[34]. Adenosine synthase A (AdsA), another sortase-anchored product, harbors a 5'-nucleotidase signature sequence. AdsA cleaves ADP or AMP to form adenosine. When inoculated into blood, *S. aureus* synthesizes large amounts of adenosine from AMP using AdsA[35]. Adenosine is a key regulator of innate and acquired immune responses and is generated at sites of inflammation, hypoxia, organ injury, and traumatic

shock[36]. Host inflammatory responses, e.g. in response to bacterial infection, are regulated to prevent excessive tissue damage during infection; production of adenosine is the key signal to end host inflammatory response[36]. Thus, staphylococci evade immune responses by signaling to the host that its inflammatory activities must be canceled.

As the pathogen has endowed itself with such a multitude of defense mechanisms, it comes as no surprise that *S. aureus*, although quickly taken up by polymorphonuclear neutrophils (PMNs) or monocytes, survives within these leukocytes[19, 37]. Gresham and colleagues demonstrated that PMNs harboring *S. aureus* can indeed seed infectious lesions when transferred into naïve mice, suggesting that staphylococci use PMNs as vehicles to enter the tissues of an infected host[38].

Stage II: preparing a site for the infectious lesion

Although staphylococci arrive within 1–3 hours in peripheral organs where abscess lesions are being established (Figure 3a), a histopathological correlate for this activity is not detectable until about 48 hours after intravenous challenge (Figure 3)[14]. At that time, sufficiently large numbers of immune cells (predominantly neutrophils) have accumulated in a small area to replace physiological epithelia with microscopically discernable lesions (Figure 3b and 3c)[14]. During Stage II of abscess maturation, early lesion sites contain a low abundance of staphylococci. It seems unlikely that the dramatic recruitment of immune cells can be explained by the massive replication of bacteria – micro- or macro-colonies of staphylococci are not observed early during infection. Molecular genetic studies on lipoprotein maturation revealed a fascinating phenotype for the *S. aureus lgt* mutant (lipoprotein diacyl-glyceride transferase), which is unable to link diacyl-glycerol to the cysteine-thiol of lipoprotein precursors[39]. The *lgt* mutant multiplied in host tissues with minimal recruitment of immune cells and did not form typical abscess lesions[40]. Thus, in addition to producing formylated peptides that are perceived by the immune system, *S. aureus* could actively release lipoproteins for recognition via Toll-like receptor 2 (TLR2)[40]. The ensuing pro-inflammatory signals could then recruit large numbers of immune cells to the site of infection, thereby contributing to the destruction of organ tissues.

Staphylococcal replication in organ tissues must meet the pathogen's nutritional requirement for iron[41]. A key pathway for the scavenging of iron at this stage involves the hemoglobin hemophore IsdB, as well as two other sortase anchored products, IsdA and IsdC, which pass the heme-moiety across the cell wall envelope for subsequent transport across the staphylococcal membrane[42]. Depending on the site of infection, too much import of heme is not a good thing. The associated production of oxygen radicals proves toxic for staphylococci and immune cells. Mutations in *S. aureus hrtA*, at the center of a pathway that perceives heme and transports superfluous heme out of bacteria, cause heme-toxicity in mutants but also interfere with neutrophil recruitment, resulting in liver-specific hypervirulence[43]. HrtA and HrtB form an ABC-type transport system that is essential for alleviating hemin toxicity. In the absence of *hrtA*, hemin toxicity triggers overexpression of the orphan permease HrtB. This triggers membrane stress, thereby augmenting the secretion of a subset of immunomodulatory proteins that are directed to inhibit neutrophil migration to the site of infection[44]. Although this is not yet known in detail, we surmise that staphylococci require several unique biosynthetic pathways during this developmental program, in particular because the pathogen must carry out its metabolism in an environment that restricts the availability of zinc[45]. SdrD, another sortase-anchored surface protein, is also required at Stage II[14]. Its specific activity during host infection is not yet known.

Stage III: crisis and disease

The molecular and cellular events that promote the formation of a staphylococcal abscess can be imagined when studying the histopathological features of maturing lesions. Within four or five days following infection, a large mass of the invading pathogen (SAC), replicates at the center of the lesion (Figure 3d and 3e). The SAC is separated from surrounding immune cells by an eosinophilic pseudocapsule that is composed of fibrin deposits[14]. This pseudocapsule is best seen by electron microscopy (Figure 4). In the periphery, staphylococcal abscesses are characterized by layers of immune cells: a ring of largely necrotic neutrophils in the immediate vicinity of SACs, followed by a layer of healthy appearing immune cells and eventually a layer of necrotic immune cells, whose border with healthy tissues is demarcated by a layer of fibrin or extracellular matrix deposits (Figure 3d and 3e)[14].

What are the molecular events that enable this astonishing metamorphosis? *S. aureus* secrete coagulase (Coa), which associates with and non-enzymatically activates prothrombin[46, 47]. The staphylocoagulase-prothrombin complex cleaves fibrinogen to generate fibrin peptides, promoting clot formation[48, 49]. One hundred years after the discovery of Coa, a second coagulase was identified[50]. von Willebrand factor-binding protein (vWbp) was initially discovered to interact with its namesake host protein, a key factor in the recruitment of platelets to blood clots[51], however vWbp also activates prothrombin to promote fibrin coagulation[52]. Although mutants lacking either *coa* or *vWbp* continue to form abscesses, variants lacking both genes were unable to form these lesions and coagulate blood (Figure 1c)[16]. Further, prothrombin, fibrin and coagulases localize to the eosinophilic pseudocapsule of staphylococcal abscesses[16]. A simple explanation for these observations is that staphylococci usurp the clotting cascade via secreted coagulases to form fibrin deposits in organ tissues. The deposits function as a barrier for immune cells preventing their penetration into SACs, where the pathogen replicates without interference while manipulating inflammatory responses, the fate of immune cells in the periphery and the progression of the entire infectious lesion from a safe distance. Additional evidence for the key contribution of coagulases to virulence and abscess formation was obtained through the discovery that *S. aureus* strains adapted to ruminants or horses require additional vWbp homologs to coagulate blood or plasma of these species and establish staphylococcal disease[53].

Mutations in two other genes affect the maturation of staphylococcal abscesses. *spa* encodes staphylococcal protein A (SpA, Figure 1a), a sortase-anchored product that is released from the bacterial envelope in large amounts. SpA impedes phagocytosis by binding the Fcγ component of immunoglobulin[54, 55], activates platelet aggregation via its binding to von Willebrand factor[56], and functions as a B-cell superantigen by crosslinking the Fab region of V_H3 bearing immunoglobulin M (IgM)[57–59]. Also, through its activation of tumor necrosis factor receptor 1 (TNFR1), SpA initiates staphylococcal pneumonia[60]. It is unknown which of the many biochemical attributes of SpA contributes to abscess formation, however, *spa* mutants appear unable to form abscesses[14]. Emp is an envelope associated protein of staphylococci, whose virulence attributes have been associated with the ability to promote adherence to endothelia[61] and biofilm formation[62]. Numerous studies have attempted to correlate staphylococcal virulence with biofilm formation; the evidence is equivocal[63, 64]. SACs are reminiscent of biofilm communities formed *in vitro*, however several genes that contribute to biofilm formation, e.g. those specifying capsular polysaccharide or poly *N*-acetylglucosamine synthesis[65, 66], are dispensable for abscess formation[14]. Immunofluorescence microscopy revealed Emp on the surface of staphylococci at the center of abscess lesions[14], suggesting that this protein contributes to abscess formation by promoting growth as biofilm-like SACs.

Stage IV: persistence and an end to the drama

Staphylococcal abscesses mature over weeks and, following rupture and release into the peritoneal cavity, lead to new infectious lesions (Stage IV). Variants lacking *eap* (extracellular adhesion protein) or a non-canonical protein secretion pathway designated Ess (ESAT-6 like secretion system) are defective in persistence and are, therefore, assigned to Stage IV[14, 67]. Ess secretion is reminiscent of the ESX-1 dependent secretion of *Mycobacterium tuberculosis*[68]. ESAT-6 and CFP10, products of the ESX-1 locus, have been shown to have immune evasive effects, including TLR2 downregulation and macrophage phagosome lysis. Deletion of the ESX-1 locus results in an attenuated strain (BCG) that forms irregular granulomas and is ultimately unable to persist within human hosts[69, 70]. Therefore, it appears that this secretion system secretes immunomodulatory factors that are required for persistence. *S. aureus* has only one ESX-1-like locus and mutations in the *ess* cluster survive stages I-III of infection but fail to persist at stage IV[67], suggesting that this pathway is also involved in prolonged staphylococcal survival within the abscess[71].

All *S. aureus* isolates appear to secrete Eap, although there is significant strain variation[72]. Structural studies showed that Eap and the C-terminal half of TSST1 (toxic shock syndrome toxin 1) as well as SEB (staphylococcal enterotoxin B) display homology, and Eap was thus shown to have superantigenic activity and to induce pro-inflammatory (IL6, TNFα) cytokine production by monocytes[73]. However, Eap has also been shown to exert numerous anti-inflammatory effects by inhibiting neutrophil adherence to the endothelium[74], leukocyte recruitment in a murine bacterial peritonitis model[75], T cell activation and recruitment to the dermis[76].

Distinctive or universal: lessons learned from *S. aureus* abscess formation

Abscesses, defined as inflammatory lesions releasing purulent material, are standard responses for many different biological, chemical or physical insults to host tissues. Using a mouse model, we describe the concept that *S. aureus* infection may modify this default program of tissue repair by building complex lesions that ensure the pathogen's persistence in a hostile environment, its dissemination throughout many different organ systems and its spread to new hosts. In support of our model we note that the injection of heat-killed staphylococci does not trigger abscess formation in mice[14]. This is unlike other biological materials, for example purified capsular polysaccharide of *Bacteroides fragilis*, that alone can activate host immune systems and promote abscess formation in a murine peritoneal challenge model[77, 78]. Further, some, but certainly not all, genes that contribute to the pathogenesis of different *S. aureus* diseases are also required for abscess formation[14]. Of those, we note that coagulases and SpA are unique to *S. aureus* isolates. A reciprocal argument is that the absence of protein A and coagulase genes from *S. epidermidis* correlates with the inability of this microbe to cause abscesses of the kind that *S. aureus* generates. The obvious conclusion for these findings is that *S. aureus* abscesses could indeed be distinctive. Whatever could perturb their formation may also benefit the outcome of infections. The benefit of such research could be significant, for example through the development of vaccines that prevent *S. aureus* skin and soft tissue infections. In accord with this, antibodies raised against either protein A or staphylococcal coagulases can each prevent abscess formation in the aforementioned mouse model[16, 79]. Future work will need to determine whether these genes are key virulence factors and vaccine antigens in other animal models of *S. aureus* infection, for example rats, guinea pigs, rabbits or non-human primates. If so, SpA and coagulases could represent important candidates for the development of a human vaccine. Recently, Kim *et al.* reported immunization of animals with a non-toxigenic variant of SpA (SpA_{KKAA}) that cannot bind Fcγ and Fab[79]. The resulting immune responses promoted opsonophagocytic clearance and protected animals against abscess formation[79]. Further, animals immunized with Spa_{KKAA} were able to mount antibody responses against

numerous *S. aureus* antigens upon challenge[79]. Typically, infection with *S. aureus* generates a modest immune response that does not afford protection against subsequent infections[79]. Thus, protective immunity against *S. aureus* infection could be achieved via the neutralization of protein A with specific antibodies.

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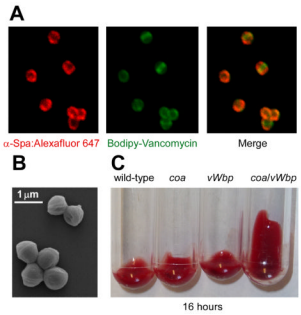

Figure 1.

Staphylococcus aureus characteristics and distinguishing features. **(a)** The immunoglobulin binding properties of staphylococcal protein A were revealed by confocal laser scanning immunofluoresence microscopy as previously described[80] . Alexafluor-647 conjugated immunoglobulin (red) binds to protein A (left), Bodipy-conjugated vancomycin (green) stains staphylococcal peptidoglycan (middle), and a merger of the two data sets reveals the deposition of immunoglobulin on the surface of staphylococci (right). **(b)** Shape and clustering of *S. aureus* cells imaged by scanning electron microscopy (SEM; image courtesy of Matt Frankel, University of Chicago). **(c)** *S. aureus* coagulates blood, a feature conferred by two secreted coagulases: staphylocoagulase (Coa) and von Willebrand factor binding protein (vWbp)[16]. Lepirudin anti-coagulated mouse blood was incubated with 10^5 cells of wild-type *S. aureus* Newman or isogenic mutants lacking *coa*, *vWbp*, or *coa*/*vWbp* and examined over time for the formation of a blood clot. Blood containing staphylococci lacking both coagulase genes do not form a clot following a 16 hour observation period.

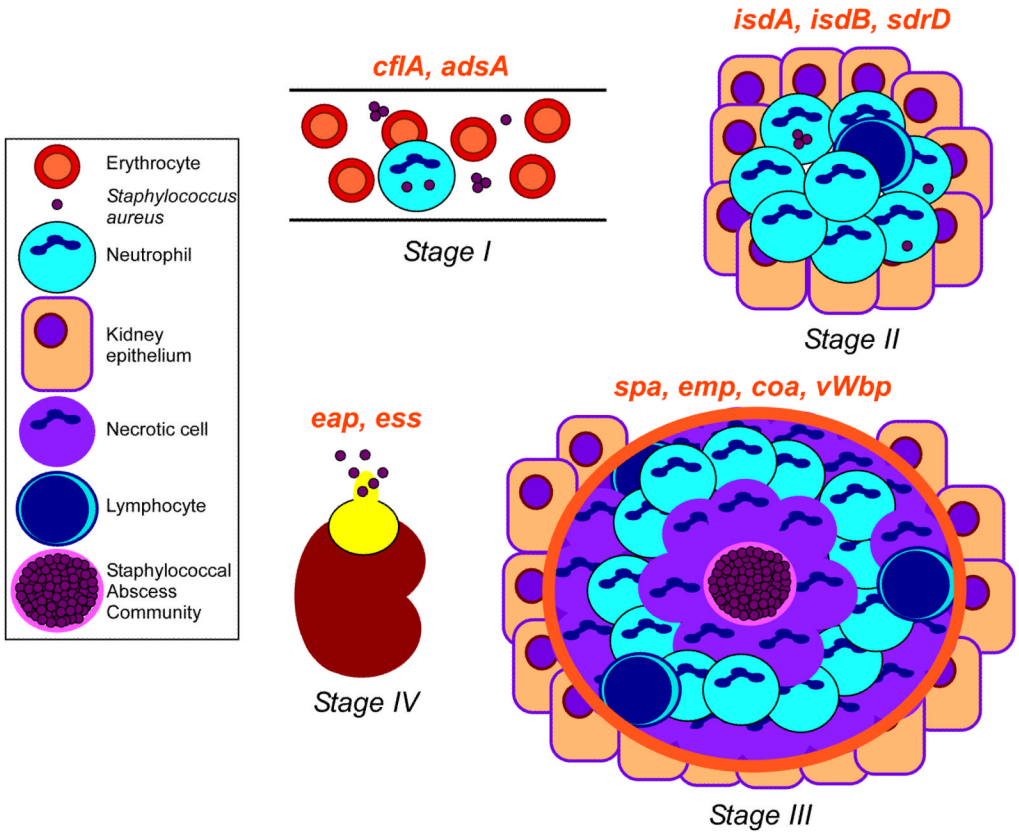

Figure 2.

Working model for staphylococcal abscess formation and persistence in host tissues. Stage I: following intravenous inoculation, *S. aureus* survives in the bloodstream and disseminates via the vasculature to peripheral organ tissues. Stage II: staphylococci in renal tissue attract a massive infiltrate of polymorphonuclear leukocytes and other immune cells. Stage III: abscesses mature showing a central accumulation of the pathogen (SAC) surrounded by a pseudocapsule of fibrin deposits (pink rim), and zones of necrotic and healthy polymorphonuclear neutrophils (PMNs; purple and light blue cells, respectively), and finally a rim of eosinophilic material (orange rim). Stage IV: abscesses mature and rupture on the organ surface to initiate new rounds of infections. Genes required for specific stages of staphylococcal abscess development are in red above the corresponding stage of infection. Figure adapted with the authors' permission from an article published by Cheng and colleagues[14].

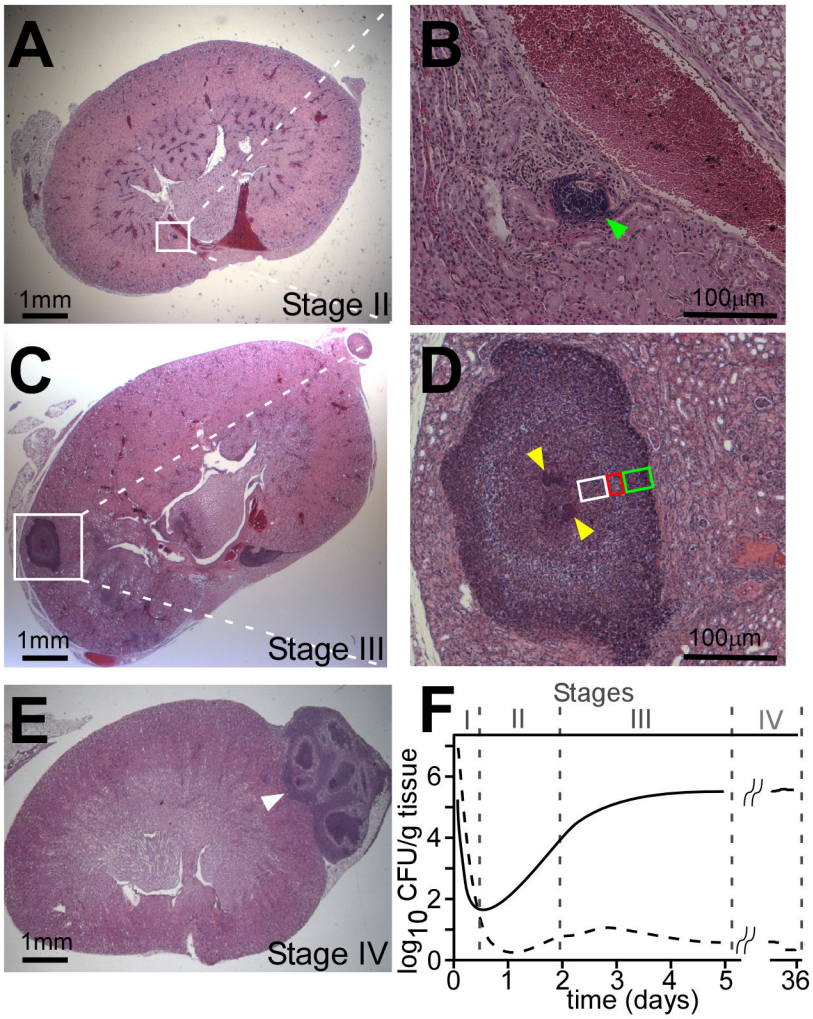

Figure 3.

Monitoring and visualizing *Staphylococcus aureus* abscess formation. **(a)–(e)** Visualization of lesions in kidneys following infection at Stages II, III and IV as previously described [14]. Paraffin embedded kidneys were thin-sectioned, stained with hematoxylineosin, mounted on glass slides and examined by light microscopy for the formation of staphylococcal abscesses. Micrographs on the left (a, c, e) show sections of entire kidneys. Micrographs on the right (b, d) represent magnifications of the area framed in the white box in (a) and (c). (b) Stage II: Site of inflammation with immune cell infiltrates (green arrowhead). (d) Stage III: staphylococci are clearly distinguishable as central nidus, staphylococcal abscess community (SAC), within the maturing abscess (yellow arrowheads). The SAC is surrounded by an amorphous, eosinophilic capsule (see Figure 4) followed by a zone of dead PMNs (polymorphonuclear neutrophils; white box), apparently healthy PMNs (red box), and necrotic PMNs (green box). The entire abscess region is separated from healthy kidney tissue by a second eosinophilic layer, which appears to increase in size over time. (e) Stage IV: Abscesses migrate to the periphery of organ tissue. The organization of SACs and immune cells during Stage III is abolished prior to rupture of the lesion onto the organ surface. **(f)** Representative distribution of bacteria in blood (dashed line) and kidney tissue (solid line) over time following intravenous infection of mice with 1

$\times 10^7$ colony forming units (CFU) of *S. aureus*. Bacterial loads are represented as CFU per g of blood or renal tissue over time following infection.

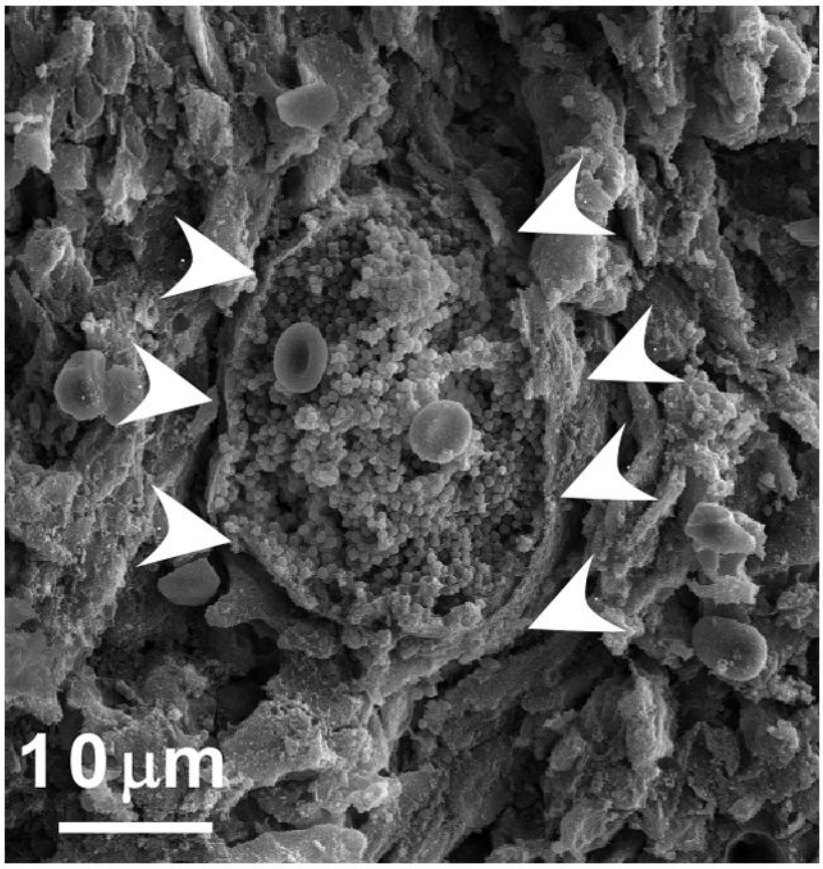

Figure 4.

SACs during Stage III. At the center of an abscess lesion, *S. aureus* cells are found as tightly associated mass covered with a granular, electron-dense substance. Bacteria are enclosed by an amorphous pseudocapsule (white arrowheads) that separates the SAC from neighboring immune cells. Image was captured from the kidney of an animal infected with *S. aureus* Newman. Organ tissue was thin sectioned, fixed, dehydrated and sputter-coated with platinum/palladium and analyzed by scanning electron microscopy.