Neutrophils Versus *Staphylococcus aureus*: A Biological Tug of War*

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Abstract

The pathogen *Staphylococcus aureus* is well adapted to its human host. Neutrophil-mediated killing is a crucial defense system against *S. aureus*; however, the pathogen has evolved many strategies to resist killing. We first describe the discrete steps of neutrophil activation and migration to the site of infection and the killing of microbes by neutrophils in general. We then highlight the different approaches utilized by *S. aureus* to resist the different steps of neutrophil attack. Various molecules are discussed in their evolutionary context. Most of the molecules secreted by *S. aureus* to combat neutrophil attacks at the site of infection show clear human specificity. Many elements of human neutrophil defenses appear redundant, and so the evasion strategies of staphylococci display redundant functions as well. All efforts by *S. aureus* to resist neutrophil-mediated killing stress the importance of these mechanisms in the pathophysiology of staphylococcal diseases. However, the highly human-specific nature of most host-pathogen interactions hinders the in vivo establishment of their contribution to staphylococcal pathophysiology.

Contents

INTRODUCTION

Staphylococcal microbes are well adapted to their host. Of all human pathogenic staphylococcal species, *Staphylococcus aureus* is the most frequent cause of disease, with skin and soft tissue infections the principal manifestations of disease (68). *S. aureus* is a human commensal; however, nasal carriage is associated with an increased risk of infection (116). Once infected with *S. aureus*, an individual may present a range of infections, from cellulitis, abscesses, and furuncles to bacteremia and sepsis, endocarditis, osteomyelitis, necrotizing fasciitis, and pneumonia (68). In the 1960s, methicillin-resistant *S. aureus* (MRSA) was identified as a nosocomial pathogen, affecting hospitalized patients with defined risk factors for acquisition (7). In the 1990s, infection of previously healthy community-dwelling individuals with MRSA was reported (109). Since then, community-acquired MRSA has rapidly emerged worldwide (113). As a result, interest in the pathophysiology of *S. aureus* has been revived. A large arsenal of *S. aureus* virulence factors, targeted at different host immune factors, has been studied. Notably, *S. aureus* effectively evades human innate immunity using virulence factors directed toward all its components (99). A great deal of these factors are directed against neutrophils or neutrophil function.

Neutrophils compose 60% of the leukocyte population present in the blood and are the most important phagocytic cells, which defend the host against acute bacterial infection. Patients with congenital neutrophil deficiencies suffer from severe infections that are often fatal (1). Diseases or disease states such as agranulocytosis, abnormality of immunoglobulins (Ig), or deficiencies of the complement system are associated with major bacterial infections. On occasion, an abnormality in neutrophil function is the underlying cause of disease. In such cases, disorders of neutrophil function (such as chronic granulomatosis disease) correlate strongly to recurrent cutaneous, periodontal, respiratory, or soft tissue bacterial infections, and in many instances *S. aureus* is the causative organism (16, 18). From such studies we can conclude that in healthy individuals neutrophil-mediated killing is the key defense system against *S. aureus*.

Indeed, as opposed to other phagocytes such as monocytes and macrophages, neutrophils are fully equipped to kill gram-positive bacteria such as staphylococci (90). This is achieved by a bombardment with reactive oxygen species (an event called metabolic burst) in combination with the armamentarium of proteases, antimicrobial peptides (AMPs), and several other enzymes (3, 17, 90). Neutrophils are therefore the most prominent cells, if not the only cells, in our immune system that effectively kill staphylococci (1). Nevertheless, staphylococci have evolved many ways to resist neutrophil-mediated killing. We first describe the sequence of events in which neutrophils travel to the site of infection and kill microbes in general. We then highlight the different strategies utilized by *S. aureus* to escape neutrophil-mediated killing.

AMP: antimicrobial peptide

NEUTROPHILS IN INFLAMMATION

Extravasation Through the Endothelium

Once hostile bacteria have successfully invaded the tissue, neutrophils leave the bloodstream and move to the site of infection. This multistep process encompasses slowing down of neutrophils on endothelial cells (i.e., rolling), firm adhesion of neutrophils, escape from the blood vessel (i.e., diapedesis), chemotactic migration, and subsequent killing of invading bacterial pathogens (**Figure 1**). To arrive at the site of entry of microorganisms, circulating phagocytes are slowed in the bloodstream in the vicinity of the infection site (115).

First, circulating phagocytes are slowed down near the site of infection. Activated endothelial cells express P-selectin and E-selectin, which interact reversibly with the glycoprotein P-selectin glycoprotein ligand-1 (PSGL-1) on the neutrophil surface (75). Owing to these loosely adhesive contacts between the phagocyte and the endothelial cells, and to the shearing force of the flow of blood, the phagocytes "roll" along the vessel wall (75). The second step is complete arrest of neutrophils and firm adherence to endothelial cells. Firm adhesion depends on the interaction between β2 integrins, such as LFA-1 and Mac-1, on the surface of phagocytes and intercellular adhesion molecule 1 (ICAM-1) molecules on endothelial cells (30). When neutrophils roll along the endothelium, interactions with chemoattractants, cytokines, bacterial products, and selectins activate and cluster the β2 integrins on the surface of the neutrophil, resulting in high-affinity interactions and subsequent firm adhesion and crawling of the phagocytes on the endothelial cells (22, 65).

Chemical mediators generated during inflammation induce and redistribute a number of complementary adhesion molecules present on the surface of both endothelial cells and neutrophils. A complex interaction between the receptors and ligands on both cells results in transmigration through the endothelial junction or even transcellularly through the endothelial cell (83) (**Figure 1**).

The leukocyte recruitment cascade described above is the generally held view of recruitment derived primarily from in vitro experiments or imaging experiments with easily accessible blood vessels. There is growing evidence that some organs do not use the same paradigm for neutrophil recruitment (84). For example, the liver does not use selectins for neutrophil recruitment and is fully dependent on hyaluronan on endothelium and CD44 on neutrophils for diapedesis (72).

Opsonization

Neutrophil phagocytosis is dependent on opsonization of the target microbe via either components of the complement system, immunoglobulins, or other innate immune components (**Figure 1**). Complement can rapidly recognize and opsonize bacteria or kill gram-negative bacteria directly by formation of the membrane attack complex (119). The human complement system consists of

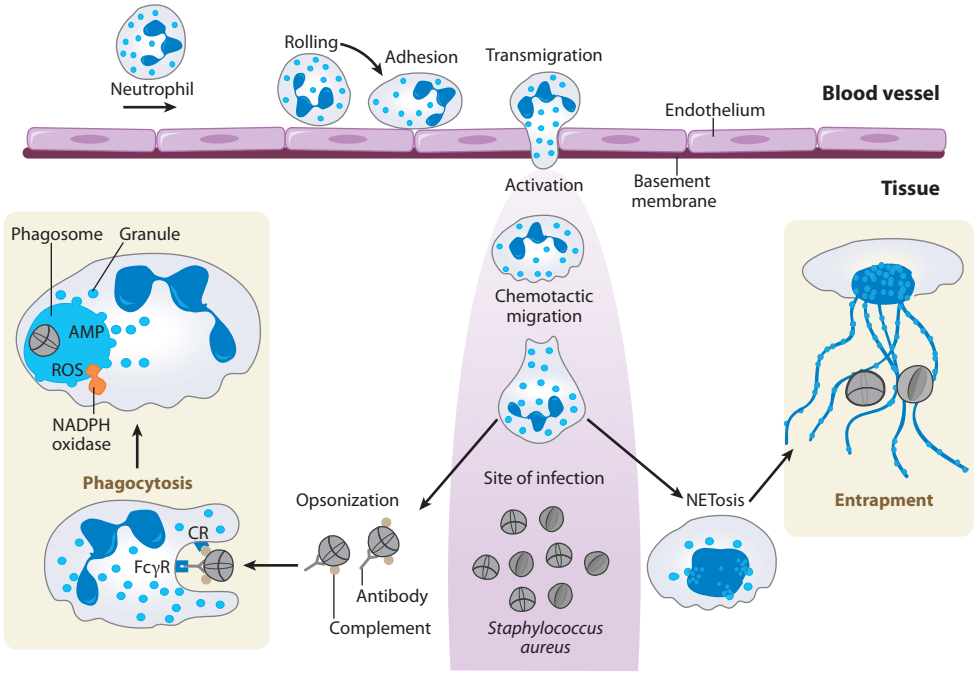

Figure 1

Neutrophil extravasation to the site of infection and killing mechanisms. Recruitment of neutrophils begins with the activation of the endothelium, followed by rolling on the activated endothelium. Upon stimulation, neutrophils stop rolling and firmly adhere to the endothelium. This step is followed by neutrophil transmigration through the endothelium into the tissue, a process known as extravasation. In tissue, neutrophils are directed by a chemotactic gradient toward the invading staphylococci, followed by recognition and subsequent phagocytosis. Phagocytosis of *Staphylococcus aureus* is dependent on the deposition of complement and immunoglobulins, which are recognized by complement receptors (CRs) and Fcγ receptors (FcγRs). Inside the neutrophil phagosome, bacteria are killed by antimicrobial proteins (AMPs) released upon granule fusion and by NADPH oxidase-derived reactive oxygen species (ROS). Alternatively, neutrophils undergo a process called NETosis, in which neutrophils expel their DNA. The DNA is decorated with histones and AMPs that extracellularly capture and kill bacteria.

more than 30 proteins and uses three independent pathways to distinguish bacteria from host cells (119). The alternative pathway is inhibited by receptors on host cells and target cells or by microbes that lack such markers. The lectin pathway recognizes distinct evolutionarily conserved structures such as polysaccharides on microbial surfaces (106). The classical pathway is initiated by antibody recognition of the microbial target and by binding of C1q to immune complexes. All three pathways converge at the formation of the C3 convertases, which are enzyme complexes that catalyze the key reaction in complement activation: cleavage of complement protein C3 into C3a and C3b (94, 99). Most of C3b is further processed into iC3b by factor H and factor I, whereas deposited C3b forms new convertases, thereby amplifying the opsonization process. Subsequently, high local concentrations of C3b induce a shift in substrate specificity of the convertase to complement protein C5. The cleavage products of C5 are C5a, a potent chemoattractant, and C5b, which initiates the lytic pathway when deposited on gram-negative bacteria. Together these complement-mediated events are responsible for the efficient detection and elimination of many different bacterial species (92).

Immunoglobulins help resolve bacterial infections via immobilization, agglutination, and opsonization, the last of which allows the recognition of immune complexes and their ingestion by phagocytes (71). Immunoglobulins activate the classical complement pathway and neutralize toxins or other bacterial virulence factors (119). Different subclasses of immunoglobulins display distinct differences in complement activation or Fcγ receptor (FcγR). For instance, IgM, owing to its polymeric nature, is particularly effective at complement activation and opsonization (123). The four IgG subclasses differ from each other with respect to their effector functions (71). This difference is related to differences in structure, notably with respect to the interactions between the variable, antigen-binding Fab fragments and the constant Fc fragment. In particular, the length and flexibility of the hinge region are different. This difference probably relates to the higher activity of IgG3 in triggering effector functions, when compared with the other subclasses (71). The capacity of the four human IgG subclasses (in monomeric form) to bind C1q decreases in the order IgG3 > IgG1 > IgG2 > IgG4, whereby IgG4 completely lacks the ability of complement activation. Effective triggering of the two major FcγRs on human neutrophils (FcγRII and FcγRIII) is mediated by both IgG3 and IgG1, resulting in phagocytosis (111).

TLR: Toll-like receptor

GPCR: G-protein-coupled receptor

FPR: formyl peptide receptor

Chemotaxis, Priming, and Activation

After crossing the endothelial barrier, neutrophils are directed toward the site of infection by a plethora of chemoattractants and then primed and activated by a variety of inflammatory stimulants (**Figure 1**). The primary activating substances are those of microbial origin. For staphylococci, the substances are primarily ligands for Toll-like receptors (TLRs) or chemoattractant receptors of the G-protein-coupled receptor (GPCR) family (6). TLRs are perhaps the best-known microbe receptors; they are responsible for recognizing a number of evolutionarily conserved structures (54). TLR ligands relevant to staphylococcal infections include bacterial lipoproteins (TLR1, TLR2, TLR6) and bacterial CpG-rich DNA (TLR9) (54). TLRs are transmembrane glycoproteins and signal through an intracellular Toll/interleukin (IL)-1 receptor (TIR) domain. Ligand-induced dimerization of TLRs is believed to trigger recruitment of MyD88 to the intracellular TIR domains to initiate signaling via the transcription factor NF-κB (49). Most TLRs form homodimers upon ligand binding. In contrast, TLR2 forms heterodimers with TLR1 (TLR1/2) and TLR6 (TLR2/6) to adequately respond to triacylated and diacylated lipoproteins, respectively (48). Gram-positive bacteria contain diacylated lipoproteins, whereas gram-negative bacteria lipoproteins may have an additional acyl group. Staphylococcal strains deficient in diacyl modification of lipoproteins fail to activate TLR2 in vivo, resulting in a reduced inflammatory response (20). Cells become activated and primed after TLR activation. TLRs assist in phagocytosis but these receptors are not involved in chemotaxis.

Neutrophils are attracted to the infection site through sensing of chemotactic factor gradients (12) (**Figure 1**). Chemoattractants are secreted by activated host cells or released as activated complement components. The chemoattractants activate phagocytes by binding to membrane-bound receptors that belong to the GPCR superfamily (12).

Newly synthesized bacterial proteins contain a formylated methionine, and as a consequence bacteria secrete a substantial amount of N-formylated proteins and peptides. fMLP, the prototype N-formyl-peptide, induces and potentiates chemotaxis, phagocytosis, and the generation of oxidative burst in neutrophils and monocytes (3). Formylated peptides act on the formyl peptide receptor (FPR1) and its homologue FPR2, both of which belong to the GPCR family. FPR1 binds fMLP with high affinity, whereas FPR2 shows low affinity for the ligand. Neutrophils express FPR1 and FPR2 but not FPR3, whereas monocytes express all three GPCRs (63).

Staphylococci produce phenol-soluble modulins (PSMs) in addition to formyl peptides. PSMs were first described as lytic molecules for neutrophils (120). PSMs not only lyse neutrophils but also activate and attract leukocytes via FPR2 (58).

PSM: phenol-soluble modulin

ROS: reactive oxygen species

Other potent chemoattractants include the small complement fragment C5a. C5a and its less potent counterpart C3a, also commonly called anaphylatoxins, are generated during the activation of the complement cascade. The formation of the small cleavage products C3a and C5a plays an important role in the attraction of phagocytes to the site of infection and in priming and activating phagocytes of gram-positive bacteria (76). In addition to bacterial fragments and complement-derived activation products, chemokines and other lipid mediators lead neutrophils to the site of infection (84).

Phagocytosis

S. aureus opsonized by complement and/or antibodies is rapidly phagocytosed upon contact with neutrophils (105). Opsonin-coated microorganisms bind to specific receptors on the phagocyte surface, and invagination of the cell membrane causes the intracellular phagosome to envelope the microorganism (56) (**Figure 1**). Uptake of the bacteria is aided by factors such as C5a; TLR ligands on the surface of the bacterium; and ligands for C-type lectin transmembrane proteins such as DC-SIGN, Dectin-1, and the mannose receptor on the surface of neutrophils (103). Moreover, phagocytosis is stimulated primarily by receptors that recognize the major opsonins, i.e., the complement factors and immunoglobulins (1).

Receptors for these opsonins, specifically the classical or leukocyte FcγRs and the complement receptors (CRs), are displayed on the neutrophil surface. Upon cross-linking by ligand binding, these receptors induce the initial uptake, or endocytosis of the pathogen, whereupon vesicular transport to lysosomes and intracellular killing are executed (1, 71, 95) (**Figure 1**).

FcγRs are members of the immunoglobulin superfamily. They contain a ligand-binding chain consisting of two or three Ig-like domains, a transmembrane region, and an intracellular domain (97). Through binding of the constant domain of IgG, FcγRs provide specificity to immune effector cells on which they are widely expressed. Cross-linking of FcγRs by IgG-opsonized particles or immune complexes induces several cell-type-dependent effector functions, including phagocytosis, respiratory burst, cytotoxicity, degranulation, and secretion of inflammatory mediators (97). On the basis of structural and biochemical differences, FcγRs are divided into three distinct classes, FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16), containing 12 isoforms that differ in cell distribution and affinity for IgG subclasses (79). Most FcγRs are activating receptors and possess immunoreceptor tyrosine-based activation motifs. FcγRIIIb is inserted into the outer layer of the plasma membrane by a glycosylphosphatidylinositol anchor and contains no signaling motif. FcγRIIa is the predominant receptor in neutrophil phagocytosis of immune complexes (112).

CR1 (CD35) binds to the active complement fragment C3b, resulting in phagocytic uptake (95) (**Figure 1**). However, CR1 is also a complement regulator with factor H activity (95). CR3 (Mac-1, CD11b/CD18) and CR4 (p150/95, CD11c/CD18) are heterodimeric glycoproteins from the integrin family with a shared β-chain (CD18). They show specificity for the iC3b fragment and bind iC3b in a Mg^{2+}-dependent fashion (108). Stimulation of neutrophils and monocytes via CR3 results in phagocytosis, production of reactive oxygen species (ROS), and release of specific granules (108). After phagocytosis and phagosome formation, neutrophils are equipped with multiple efficient mechanisms aimed to kill intracellular bacteria.

NET Formation

In addition to phagocytosis and intracellular killing, neutrophils evolved a recently discovered defense strategy whereby their nuclear contents are expelled together with several cytosolic and

granular proteins. This results in the formation of neutrophil extracellular traps (NETs), which trap and thereby prevent further dissemination of the pathogens (19). Although some essential steps in the formation of NETs (NETosis) have been described (66), the mechanisms for their initiation, downstream activation pathways, and effector functions are still under investigation. In vitro activation of NETs can be initiated by different stimuli such as phorbol myristate acetate, lipopolysaccharide, IL-8, and different bacteria (85, 117). However, Yipp et al. (124), using intravital microscopy, show that in vivo neutrophil NETosis is tightly regulated through TLR2 and complement-mediated opsonization. NETs can trap microbes ex vivo and can be observed in animal models of *S. aureus* infection (73). In the bloodstream, the concentration of AMPs is too low to be effective in killing microbes; however, when associated with NETs, AMPs reach their critical antimicrobial concentrations (19).

NET: neutrophil extracellular trap

Bacteria Killing by Means of Neutrophil Granules

Neutrophils are end-stage cells that store most of their toxic antimicrobial compounds in ready-to-use granules (17), which are available during encounters with bacteria, are safe for storage, and support the dynamic interplay between microbes and the associated inflammatory and microbe-killing responses of immune cells (3). Several enzymes target the bacterial cell wall (e.g., lysozyme), function as proteases to degrade bacterial toxins (e.g., neutrophil elastase), or assist in metabolic burst (myeloperoxidase, MPO, associated with phagocytosis) (56). A group of directly bactericidal and bacteriostatic proteins and peptides primarily attack the bacterial membrane [e.g., the bactericidal/permeability-increasing (BPI) protein] or interfere with the cell wall synthesis machinery (e.g., defensins) (28). Different granules fulfill different purposes in the complex dynamics involving neutrophil migration, phagocytosis, and killing. The membrane or secretory vesicles, which are located near the plasma membrane and are very rapidly secreted, serve as a reservoir for a number of important membrane-bound molecules and receptors employed during neutrophil migration and the early steps of opsonic recognition (17). Azurophilic granules contain many antimicrobial proteins such as MPO; BPI; lysozyme; defensins; and the serine proteases neutrophil elastase, proteinase 3, and cathepsin G. Secondary granules contain lactoferrin, NGAL, hCAP-18, and lysozyme. Tertiary granules contain a number of metalloproteases, including gelatinase (1, 17). Chelating agents interfere with microbial metabolism and are strongly bacteriostatic. Lactoferrin binds preferentially to iron, and calprotectin chelates zinc (26). However, calprotectin is not stored in granules. In neutrophils, as opposed to all other cells, calprotectin constitutes more than 60% of the proteins in the cytosol (26, 122).

Bacteria Killing by Reactive Oxygen Species

Neutrophil activation leads to the production of ROS, a process known as oxidative burst. ROS represent a complex and dynamic mixture of highly reactive molecules that differ in their stability in, reactivity to, and permeability through membranes, but all can severely damage microbes (42). In the small space between an ingested bacterium and the membrane of the phagosome, molecular oxygen is reduced and high levels of superoxide are generated upon assembly of the NADPH-dependent oxidase (3). Electrons are pumped into the phagosome, which is compensated for by an influx of protons or other cations. The protons are used to reduce superoxide to hydrogen peroxide (3). Hydrogen peroxide associates with chloride to form hypochlorous acid in a reaction catalyzed by MPO. Hydroxyl radical, chloramines, hydroperoxyl radical, peroxynitrite, and singlet oxygen, all very effective antimicrobial compounds, are generated in secondary reactions

(56). Augmentation of the NADPH oxidase by MPO facilitates a most efficient response against invading microorganisms, and this property is unique for neutrophils (3).

SSL: staphylococcal superantigen-like

Taken together, neutrophils are highly efficient at finding, recognizing, phagocytosing, and destroying bacterial invaders such as *S. aureus*. Thus, bacteria that cannot defend themselves become passengers and targets for neutrophil responses that protect hosts from bacterial invaders. Below, we summarize the different ways in which *S. aureus* fights neutrophil attacks and explain how this pathogen addresses each of the discrete defense mechanisms implemented by neutrophils.

STAPHYLOCOCCAL NEUTROPHIL EVASION

Evading Extravasation

The first step of extravasation, neutrophil rolling on endothelial cells (**Figure 1**), is modulated by staphylococcal superantigen-like 5 (SSL5), which acts on P-selectin glycoprotein ligand-1 (PSGL-1) (13). By binding PSGL-1, SSL5 blocks its interaction with the natural ligand P-selectin and abrogates neutrophil rolling on endothelial cells (13). SSL5 binding to PSGL-1 is glycan dependent. X-ray crystallography data revealed the structure of SSL5 in complex with sialyl Lewis X (4), the predominant carbohydrate decoration on PSGL-1. Further, SSL5 is also thought to scavenge chemokines from chemokine receptors by binding to other glycoproteins (13). All chemokines contain a highly homologous glucosaminoglycan (GAG)-binding site, which allows their presentation by endothelial cells or extracellular matrix. SSL5 targets the binding site, common among all chemokines, thereby inhibiting the activation of integrins that promote neutrophil attachment (14).

The second step in neutrophil extravasation, the firm adhesion of neutrophils to the endothelium, is also targeted by *S. aureus*. ICAM-1, the crucial molecule for this interaction, is bound and inhibited by extracellular adherence protein (Eap) (25), blocking also the final molecular adherence step in primary rolling and transmigration events in the bloodstream near the infection site. The function of these modulators is illustrated in **Figure 2*a***.

Evading Chemotaxis, Priming, and Activation

S. aureus secretes many proteins that affect chemokine signaling (**Figure 2*b***). In addition to inhibiting PSGL-1, SSL5 inhibits chemokine-induced leukocyte activation in a different way. SSL5 inhibits leukocyte responses to chemokines CXC, CC, and CX3C and to the complement fragments C3a and C5a (12). SSL5 directly binds the N termini of chemokine receptors in a glycan-dependent manner. SSL5 also binds to the FPR1, the FPR2, the leukotriene B4 receptor, the platelet-activating factor receptor, and the nucleotide receptor P2Y2 (14). However, SSL5 does not appear to interfere with the activation of these receptors, as nonproteinaceous signals are sensed via the transmembrane domains of GPCRs. The nonprotein stimuli interact primarily with the transmembrane regions of their target GPCR. Chemokines bind the N terminus of their receptors and subsequently interact with the pocket formed by the transmembrane domains. Thus, SSL5 targets the glycosylated N termini of GPCRs and inhibits only stimuli that require the receptor N terminus for activation (14).

SSL10 inhibits cell responses mediated by the chemokine CXCL12 (or stromal cell-derived factor-1 α, SDF-1α). When cells are treated with SSL10, calcium mobilization and chemotaxis, in response to CXCL12, are abrogated (118). CXCL12-induced intracellular signaling was also affected in cells pretreated with SSL10. SSL10 is thus specific for CXCR4-mediated responses, as it did not inhibit CXCL8- or C5a-induced calcium responses (118).

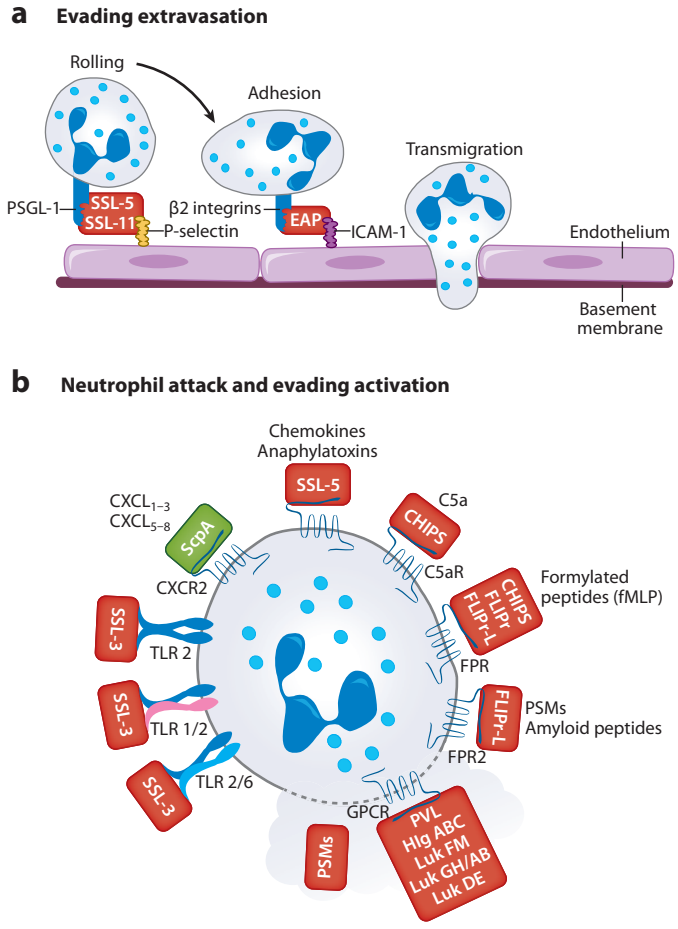

Figure 2

Evasion of neutrophil extravasation, activation, and neutrophil attack. (*a*) Mechanisms by which *Staphylococcus aureus* subverts neutrophil extravasation. (*b*) Neutrophil attack and evasion of activation. Red boxes represent an antagonizing protein of *S. aureus*, and green boxes represent a protease of *S. aureus*. Abbreviations: PSGL-1, P-selectin glycoprotein 1; SSL, staphylococcal superantigen-like protein; ICAM-1, intracellular adhesion molecule 1; EAP, extracellular adherence protein; TLR, Toll-like receptor; GPCR, G-protein-coupled receptor; C5aR, C5a receptor; CXCR, chemokine receptor; CHIPS, chemotaxis inhibitory protein of *Staphylococcus*; FPR, formyl protein receptor; FLIPr and FLIPr-L, FPR-like inhibitory proteins; PSMs, phenol-soluble modulins; ScpA, Staphopain A; LUK, leukocidin; Hlg, hemolysin-gamma; PVL, Panton-Valentine leukocidin.

Proteases also assist in chemokine signaling inhibition. Neutrophils treated with Staphopain A are unresponsive to activation by CXCR2 chemokines due to cleavage of their N-terminal domain (59). Moreover, Staphopain A inhibits neutrophil migration toward CXCR2 chemokines. By comparing a wild-type MRSA strain with an isogenic Staphopain A mutant, it was demonstrated that Staphopain A is the only secreted protease with activity toward CXCR2 (59). Secretion of Staphopain A at the site of infection is likely to inhibit neutrophil activation and recruitment, adding to the elaborate immune evasion repertoire of *S. aureus* (59).

CHIPS: chemotaxis inhibitory protein of *Staphylococcus*

FLIPr: FPR-like 1 inhibitory protein

CHIPS (chemotaxis inhibitory protein of *Staphylococcus*), a small protein of 14.1 kDa, binds and inhibits FPR1 as well as C5aR. CHIPS impairs neutrophil chemotaxis (40). The CHIPS-binding site on FPR1 involves multiple regions in the extracellular domains of the receptor. CHIPS probably binds near or directly at the fMLP-binding pocket, since fMLP is a small peptide that would otherwise easily bypass the blockade. The N terminus of CHIPS is important for its activity toward FPR1 (39).

CHIPS exerts its effect on C5aR through binding to amino acids 10 to 18 in the N terminus of this receptor (88). Deletion of the first 30 amino acids does not affect its C5aR-inhibiting activity, suggesting two different active sites. The 3D structure of the C5aR-blocking domain of CHIPS (CHIPS31-121) was revealed by X-ray crystallography (40). CHIPS31-121 is composed of an α-helix packed into a four-stranded antiparallel β-sheet. This domain is structurally homologous to the C-terminal domain of SSL proteins (2).

A search for *S. aureus* proteins homologous to CHIPS led to the identification of FPR-like 1 inhibitory proteins (FLIPr and FLIPr-like). As outlined above, formyl peptides provide a bacteria-specific broad signature that is sensed by formyl peptide receptors. *S. aureus* has evolved at least two antagonists for the FPRs. Both CHIPS and FLIPr-like inhibit FPR1 (86), thereby avoiding recognition via this unique recognition pathway. FLIPr and FLIPr-like also inhibit FPR2 and thus evade recognition of the PSMs that are secreted by staphylococci (58, 87).

Together these molecules inhibit the first chemoattractants from migrating toward the site of infection (59, 40, 88). Furthermore, cellular activation, important for cosignaling events during phagocytosis, is inhibited. FLIPr and FLIPr-like also impair responses to FPR2 agonists; they inhibit neutrophil calcium mobilization, actin polymerization, and/or chemotaxis to the synthetic peptides of endogenous proteins serum amyloid A, amyloid β1, and prion protein. In *S. aureus* biofilms, PSMs form amyloid-like fibers (98). Although these fibers are different from monomeric PSM, it is tempting to speculate that FPR2 may be involved in amyloid recognition.

TLRs are crucial for host defense against microbial infections. TLR2 is especially important as it, together with TLR1 and TLR6, recognizes bacterial lipoproteins of both gram-positive and gram-negative origin. Present on a variety of immune cells, TLR2 is critical for host protection against *S. aureus*. SSL3 specifically binds and inhibits TLR2 activation on human and murine neutrophils and monocytes. Through binding of the extracellular TLR2 domain, SSL3 inhibits IL-8 production by HEK cells expressing TLR1/2 and TLR2/6 dimers, stimulated by their specific ligands. The SSL3-TLR2 interaction is partially glycan dependent (5, 125). This unique function of SSL3 adds to the arsenal of evasive molecules that *S. aureus* deploys to subvert both innate and adaptive immunity.

Evading Opsonization, Phagocytosis, and NET Formation

S. aureus has evolved an entire array of highly specific complement-modulating strategies as illustrated in **Figure 3**. The secreted factors described below allow bacteria to either diminish or delay the detrimental effects of an innate immune attack, thereby generating a window of opportunity to replicate and establish a microenvironment conducive to bacterial survival and disease pathogenesis.

The secreted metalloprotease aureolysin attacks the central molecule in the complement system, C3. It effectively inhibits phagocytosis and killing of bacteria by neutrophils. Strikingly, in contrast to other proteases, aureolysin is more active in serum than in isolated form. Aureolysin cleaves purified C3 specifically in the α-chain, close to the C3 convertase cleavage site (which differs by two amino acids), yielding active C3a′ and C3b′ (60). However, in serum the aureolysin-generated C3b is further degraded by factor H and factor I. Using an aureolysin mutant of *S. aureus*

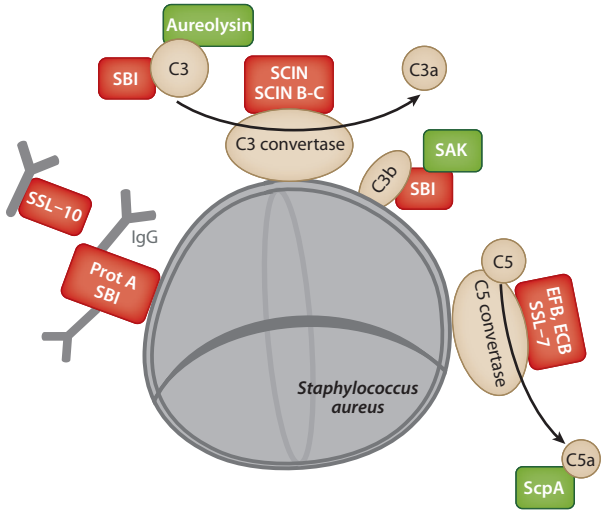

Figure 3

Evasion of opsonization. Schematic representation of the molecular mechanisms of staphylococcal inhibition of opsonization and subsequent phagocytic uptake by neutrophils. Red boxes indicate an antagonizing protein, and green boxes indicate a staphylococcal protease. Tan circles and ovals indicate host complement proteins. Abbreviations: SBI, staphylococcal binder of IgG; SSL, staphylococcal superantigen-like protein; IgG, immunoglobulin G; SAK, staphylokinase; SCIN, staphylococcal complement inhibitor; ECB, extracellular complement-binding protein; EFB, extracellular fibrinogen-binding protein; ScpA, Staphopain A.

USA300, Laarman et al. (60) showed that aureolysin is essential and sufficient for C3 cleavage by bacterial supernatant. Aureolysin acts synergistically with host regulators to inactivate the central complement component C3, thereby dampening host immune responses (60).

The staphylococcal complement inhibitor (SCIN) is a 10-kDa excreted protein that blocks all complement pathways: the lectin, classical, and alternative pathways. SCIN (and SCIN-B and SCIN-C) efficiently blocks phagocytosis and killing of staphylococci via inhibition of C5a production (94). SCIN specifically acts on surface-bound C3 convertases, with two major consequences. First, SCIN stabilizes C3bBb at the surface of the bacterium. Second, binding of SCIN to C3bBb impairs the enzymatic activity of the convertases (91). The crystal structure of the C3 convertase formed by C3b, Bb, and SCIN was dimeric in nature. SCIN blocks the formation of a productive enzyme-substrate complex (36, 94). Formation of dimeric convertases by SCIN is important for *S. aureus* immune evasion because it modulates complement recognition by phagocytic receptors. Dimeric, but not monomeric, SCIN convertases showed impaired binding to CR1 and the complement receptor of the immunoglobulin superfamily. The dimerization site of SCIN is essential for its strong antiphagocytic properties (52).

The extracellular fibrinogen-binding (Efb) molecule is a 15.6-kDa excreted molecule that was initially described to associate with fibrinogen. Efb also binds the C3d region of C3 (64). Efb and extracellular complement-binding (Ecb) protein can modulate the alternative pathway convertase by directly binding to the C3b molecule (51). The crystal structures of both molecules in complex with the C3d domain of C3 revealed their exact binding sites (41). Compared with wild-type staphylococci, targeted inactivation of the genes encoding Ecb and Efb strongly attenuates *S. aureus* virulence in a murine infection model: Mice experienced significantly higher mortality rates upon intravenous infection with wild-type bacteria than with an isogenic ΔEcbΔEfb mutant. Ecb and Efb are also required for staphylococcal persistence in host tissues and abscess formation

SCIN: staphylococcal complement inhibitor

in the kidneys. In a pneumonia model, Ecb and Efb together promote bacterial survival and block neutrophil influx into the lungs. Thus, Ecb and Efb are essential to *S. aureus* virulence in vivo and could represent attractive targets for vaccine development (53).

Similar to other successful bacterial pathogens, *S. aureus* recruits the complement regulatory protein factor H to its surface to inhibit the alternative pathway of complement. Studies using recombinant SdrE revealed binding to factor H as well as factor I–mediated cleavage of C3b to iC3b (100).

SSL7 binds IgA and complement C5, thereby inhibiting IgA-FcαRI binding and serum killing of *Escherichia coli* (61). Furthermore, SSL7 inhibits the generation of C5a induced by staphylococcal opsonization, which is slightly enhanced by its IgA-binding capacity. SSL7 has strong protective activity against staphylococcal clearance in human whole blood. SSL7 strongly inhibited the C5a-induced phagocytosis of *S. aureus* and oxidative burst in an in vitro whole-blood inflammation model (11). The crystal structure of the C5-SSL7 complex confirms that binding to C5 occurs exclusively through the C-terminal beta-grasp domain of SSL7, leaving the oligosaccharide-binding domain free to interact with IgA (62).

The first antiopsonic molecule of *S. aureus* to be described was staphylococcal protein A (SpA), which can bind the Fc part of IgG (33). SpA is linked to the cell wall of *S. aureus* via its sorting signal and sortase. SpA is released from the staphylococcal cell wall into the extracellular medium during growth. Through binding of IgG, SpA blocks FcR–mediated phagocytosis and is a highly efficient complement activation modulator by interfering with the binding of C1q (31). SpA was also described as a B cell superantigen, promoting B cell activation. Subsequent analysis revealed that surface SpA, similar to B cell receptor cross-linking with antihuman immunoglobulin, sensitizes B cells for the recognition of cell wall–associated TLR2-active lipopeptides (8). Immunization of mice with SpA, mutated in each of the five immunoglobulin-binding domains, raised antibodies that blocked the virulence of staphylococci, promoted opsonophagocytic clearance, and protected mice against challenge with highly virulent MRSA strains (55).

In addition to its properties as a CXCR4 antagonist, SSL10 binds IgG with consequences for FcR recognition and complement activation. The specific interaction between recombinant SSL10 and human IgG has been confirmed by far-Western blot analysis, pull-down analysis, and surface plasmon resonance, revealing a dissociation equilibrium constant of 220 nM (46).

Two staphylococcal proteins affect both immunoglobulin and complement. The staphylococcal IgG-binding molecule Sbi has two IgG-binding domains, similar to SpA (33). Sbi-III and Sbi-IV can also bind to C3, inhibiting activation (21). Furthermore, Sbi binds the human complement regulators factor H and factor H–related proteins and can form a stable tripartite complex with C3 and factor H (43). Together these actions result in inhibition of the alternative pathway (43).

Staphylokinase (SAK) recruits plasminogen to the staphylococcal surface and activates the zymogen to form active protease. Plasmin cleaves human IgG as well as human C3b and iC3b from the bacterial cell wall, leading to impaired phagocytosis by human neutrophils. Plasmin removes the entire Fc fragment, thereby inhibiting the activation of the classical pathway of complement and FcR recognition. Plasmin cleaves C3b in both α- and β-chains (93).

All the proteins described above influence opsonization and therefore phagocytic uptake. NET formation is also inhibited by a secreted staphylococcal nuclease. The isogenic nuclease-deficient *S. aureus* mutant was impaired in its ability to degrade NETs in vitro. Also, the mutant strain was more susceptible to extracellular killing by activated neutrophils. Moreover, *S. aureus* nuclease production was associated with delayed bacterial clearance in the lung and increased mortality after intranasal infection. Therefore, it is likely that the *S. aureus* nuclease promotes resistance against NET-mediated antimicrobial activity of neutrophils and contributes to disease pathogenesis in vivo (10).

Evading Killing

S. aureus strains shield themselves against ROS via their golden pigment, staphyloxanthin, which functions as an antioxidant and provides resistance to killing by peroxide and singlet oxygen (67). In agreement with this model, a staphyloxanthin mutant, unlike wild-type staphylococci, cannot survive within neutrophils. When the oxidative burst of neutrophils is inhibited with DPI (diphenyleneiodonium), the difference in survival between wild-type and staphyloxanthin mutant staphylococci is lost (67). Thus, staphyloxanthin at least partly blocks the ROS activities of neutrophils.

In addition to staphyloxanthin, several staphylococcal enzymes contribute to resistance against ROS. The main enzyme in the staphylococcal cytoplasm for removing oxidative stress is catalase (70). Catalase removes hydrogen peroxide by converting the compound to oxygen and water, thereby protecting the phagocytosed staphylococci. *S. aureus* further harbors the alkyl hydroperoxide reductase gene (*ahpC*), which encodes for an enzyme with catalase activity. Recently, yet another protein, SOK (surface factor promoting resistance to oxidative killing), was described that also confers resistance to neutrophil killing. SOK is exposed at the extracellular surface. A SOK mutant has an increased sensitivity to singlet oxygen. In an in vitro phagocytosis model the wild type is more resistant to killing by neutrophils compared with the SOK mutant strain (69). Importantly, catalase activity provided by either KatA or AhpC is required for normal growth under aerobic conditions (27), and as such its additional effects on immune evasion after phagocytosis can be an added value, if not the main target of these enzymes.

Lysozyme degrades the cell wall peptidoglycan matrix by breaking the β-1,4 glycosidic bonds between *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlucNAc), causing lysis of the bacteria (96). However, *S. aureus* is insensitive to lysozyme. The *O*-acetyltransferase A (OatA) enzyme of *S. aureus* causes O-acetylation of the peptidoglycan, and therefore the muramidase activity of lysozyme is no longer capable of degrading its peptidoglycan (9, 44).

S. aureus has at least two independent mechanisms to resist the attack of defensins that disrupt the integrity of the bacterial cell wall. First, the cell membrane itself is modified. The underlying mechanism for this resistance is a modification of phosphatidylglycerol with L-lysine. This modification leads to a reduced negative charge of the membrane surface, likely repelling the cationic peptides. An *mprF* mutant strain killed considerably faster by human neutrophils exhibited attenuated virulence in mice (81, 82). Second, *S. aureus* secretes staphylokinase, which activates host plasminogen. Direct binding between α-defensins and staphylokinase nearly inhibits the bactericidal effect of α-defensins. Staphylokinase with a blocked plasminogen-binding site still retained its ability to neutralize the bactericidal effect of α-defensins (50). Carotenoid pigments play a role in resistance against cationic AMPs. An increase in carotenoid rigidifies the cell membrane, making it less susceptible to the AMPs (74).

A metalloprotease secreted by *S. aureus*, aureolysin, is capable of cleaving LL-37, one of the few AMPs with potent activity against staphylococci (101). Finally, *S. aureus* resistance against AMPs is mediated by positive-charge modifications of the cell wall through the incorporation of cell wall teichoic acids and lipoteichoic acids (121). An overview of these protective factors is provided in **Figure 4**.

Neutrophil Attack

By secreting cytolytic toxins, *S. aureus* protects itself against killing by the host immune system both before and after engulfment by neutrophils, as shown in **Figure 2*b***. β-Barrel pore-forming toxins target the cell membrane and induce leakage and ultimately lysis of eukaryotic cells. The

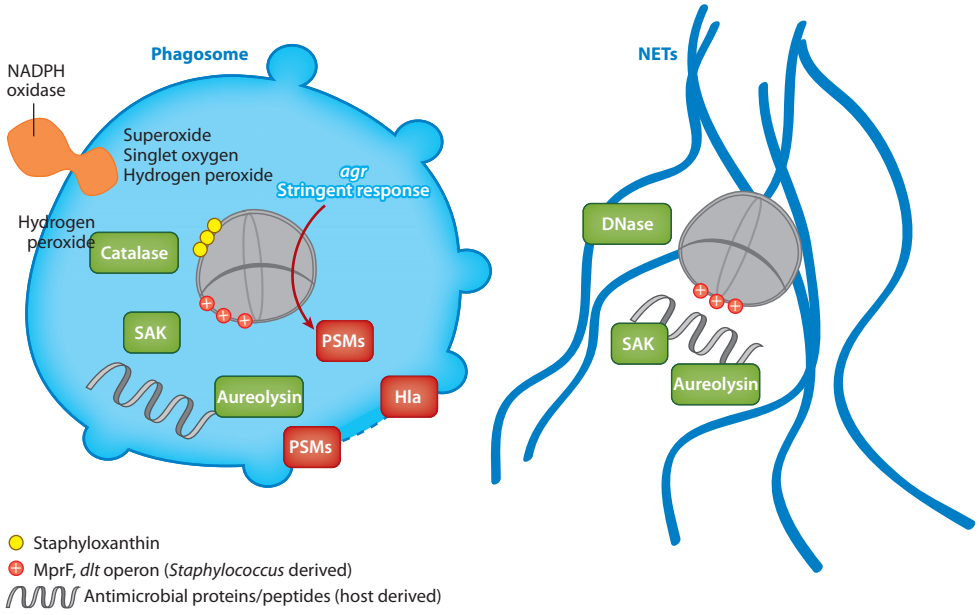

Figure 4

Evasion of neutrophil killing. Schematic representation of the mechanisms utilized by *Staphylococcus aureus* to avoid killing inside neutrophils or by neutrophil extracellular traps (NETs). Staphyloxanthin provides an antioxidant shield, and catalase detoxifies hydrogen peroxide. Resistance to cationic antimicrobial peptides (AMPs) is mediated by positive-charge modifications of the cell wall, aureolysin-mediated proteolysis, and binding/inactivation by staphylokinase (SAK). Induction of the stringent response and the quorum-sensing mechanism *agr* leads to intraphagosomal phenol-soluble modulin (PSM) and hemolysin-alpha (Hla) production and thereby disruption of the phagosomal membranes. Staphylococcal DNase can cleave the DNA backbone of NETs. Red boxes indicate an antagonizing protein, and green boxes indicate a staphylococcal protease. Abbreviations: *agr*, accessory gene regulator; MprF, multiple peptide resistance factor.

hemolysin-alpha, also known as α-toxin, is secreted as a monomer and associates as a homomultimeric pore in the membrane of the host target cells (15). Although hemolysin-alpha is not cytotoxic to neutrophils, it lyses other immune cells such as macrophages, lymphocytic subpopulations, and erythrocytes (110). Hemolysin-alpha was recently reported to bind and upregulate the epithelial zinc-dependent metalloprotease ADAM-10 (45).

The bicomponent β-barrel pore-forming leukocidins comprise two subunits that are independently secreted as monomers and form heteromultimeric pores in the membrane of the host target cells (47). Currently, five leukocidins of *S. aureus* have been described: Panton-Valentine leukocidin (PVL), hemolysin-gamma (Hlg), leukocidin E/D (LukED), leukocidin G/H (LukGH), and leukocidin M/F-like (LukMF). The *hlg* and *lukED* genes are present in the chromosome of 99% of all *S. aureus* isolates, whereas the genes encoding PVL are located on bacterial prophages and are found in only 2% of all isolates; however, the majority of community-acquired MRSA isolates carry the genes encoding PVL (77). Further investigations revealed a clear species specificity of PVL cytotoxicity, in which mice and macaque neutrophils were resistant, whereas human and rabbit neutrophils were susceptible. The species specificity of these bicomponent toxins is determined most likely by the interaction with host GPCRs, as demonstrated for LukED (35) and PVL (102). By targeting GPCRs, *S. aureus* has a highly selective tool to attack phagocytes before it is engulfed.

The recently discovered potent cytolytic peptides, PSMs, have a common amphipathic α-helical region, which is thought to enable their ability to lyse cells most likely by disrupting the cell membrane. Despite having a common structure, PSMs are categorized in two groups depending on their size. The shorter α-type PSMs (20–30 amino acids long) are more toxic than the β-type PSMs (approximately 44 amino acids long). In vivo infection and inflammation models showed a pronounced role for α-type PSMs in staphylococcal pathogenesis (120).

Escape from Neutrophils

Multiple lines of evidence accumulated in recent years have demonstrated that *S. aureus* can survive within host cells and use host cells as vehicles for its dissemination from the site of infection (107). In particular, community-acquired MRSA strains have an enhanced capacity to lyse neutrophils after phagocytosis, leading to increased bacterial survival (57). The concentration of AIP, responsible for gene transcription activation of the staphylococcal quorum-sensing mechanism *agr*, can reach critical activating levels within cells. This allows the *agr* system to function within neutrophils (23), resulting in upregulation of PSMs and in cellular lysis. In line with these studies, the stringent response, characterized by the rapid synthesis of (p)ppGpp as messenger of environmental stress conditions, seems to precede the quorum-sensing mechanism (37). Both systems are crucial for upregulating PSMs intracellularly and mediating the lysis of neutrophils after phagocytosis and subsequent escape of *S. aureus* (37). This finding is further supported by the notion that PSMs are rapidly inactivated by serum lipoproteins, suggesting that these molecules are unlikely to function in extracellular environments (104, 105). However, the importance of single virulence factors contributing to intracellular survival seems to be dependent on the type of host cell and bacterial strains analyzed. For example, in the strain USA300 LAC, LukGH may have a role in intracellular lysis (32, 114). Furthermore, δ-toxin, β-toxin, and β-PSMs have important roles in staphylococcal escape from phagoendosomes of human epithelial and endothelial cells (38).

CONCLUSION AND OUTLOOK

Worldwide, *S. aureus* has been and still is a prominent cause of bacterial infections in humans (31, 68). Over the past few decades, *S. aureus* has become highly effective at acquiring resistance to antibiotics (24). The emergence of hospital-acquired and community-acquired MRSA has now become a global problem (29). Besides antibiotic resistance, enhanced virulence of community-acquired MRSA strains seems to explain the successful spread and increased incidence of infections (80, 89). The large number of infections takes a toll on public health systems, causing significant financial burden (68). Owing to the acquisition of resistance, many inexpensive, nontoxic, and previously highly effective antibiotics can no longer be used as first-line therapy for *S. aureus* infections. Because no new antibiotic agents are expected to be released in the near future (29), the outlook for this current public health threat is grim. A promising staphylococcal vaccine recently failed in clinical trials, and no new vaccine candidates are expected in the short term (34). To overcome the impasse described above, new approaches are urgently needed. One alternative approach is to gain a better understanding of the host-pathogen interaction and to target the host and pathogen factors involved.

As described in the nineteenth century by Sir Alexander Ogston, the neutrophil is a central player in the interaction between host and *S. aureus* (78). Over the last decade, multiple mechanisms by which *S. aureus* successfully counteracts attack by neutrophils were described. The first mechanism, discussed in this review, is the potential of *S. aureus* to counteract the host response at nearly all levels, allowing it to employ its armament depending on local infection dynamics.

The extensive number of strategies stresses the importance of neutrophil escape in staphylococcal pathophysiology. The second mechanism is the specificity for human targets of most molecular mechanisms utilized by *S. aureus*. CHIPS, SCIN, SAK, many leukocidins, including PVL, and most of the SSLs are human specific in their mechanism of action. The specificity can be explained by high-affinity protein-protein interactions not consistently compatible with other species. This extensiveness, together with the human specificity of the strategies *S. aureus* evolved to overcome neutrophil attack, offers challenges for the near future. By focusing on the interaction of one molecule with its compatible host counterpart, the contribution of other relevant but noncompatible molecules is prone to neglect. An ideal model for staphylococcal disease, other than the human model, probably does not exist. Results obtained from experimental models for infection, most notably in rodents, should therefore be interpreted with caution.

The need for new treatment options, both preventative and curative, is high. A better understanding of the critical tug of war between *S. aureus* and the neutrophil might offer leads for the development of new drugs and vaccines.

SUMMARY POINTS

1. Neutrophils are crucial for host defense against infections with *S. aureus*.
2. Neutrophils are well equipped to destroy staphylococci, both intra- and extracellularly.
3. Neutrophil recruitment, chemotaxis, priming, and activation are all multistep processes.
4. *S. aureus* targets the neutrophil-mediated host defense at all levels.
5. Most of the strategies employed by *S. aureus* show clear human specificity.
6. High-affinity protein-protein interactions often cause human specificity.
7. The extensive repertoire of staphylococcal evasion mechanisms reflects their importance in pathophysiology.
8. The interaction between *S. aureus* and its human host hinders comprehensive studies in experimental models for disease.

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Errata

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