



## REVIEW ARTICLE

# Staphylococci evade the innate immune response by disarming neutrophils and forming biofilms

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*Staphylococcus aureus* and *Staphylococcus epidermidis* can cause many types of infections, ranging from skin infections to implant-associated infections. The primary innate immune response against bacterial infections involves complement activation, recruitment of phagocytes (most importantly neutrophils), and subsequent killing of the pathogen. However, staphylococci are not innocent bystanders; they actively obstruct this immune attack. To do that, *S. aureus* secretes several immune-evasion proteins to resist attack by the innate immune system. Furthermore, *S. aureus* and *S. epidermidis* are known for their ability to form biofilms on implanted medical devices and host tissues, which provides another important immune-evasion mechanism. Understanding these different strategies to resist immune attack will help to develop novel therapies against staphylococcal infections.

**Keywords:** antibodies; biofilm; complement; evasion; neutrophils; *S. aureus*; *S. epidermidis*; Staphylococci

Staphylococci, most importantly *Staphylococcus aureus* and *Staphylococcus epidermidis*, are a frequent cause of nosocomial infections. They can cause a broad range of infections, from acute diseases, such as skin abscesses and sepsis, to chronic infections such as implant-related infections [1–3]. Neutrophils are key players in the host defense against staphylococci. They move to the site of infection to engulf bacteria via phagocytosis and kill them intracellularly. Antibodies and complement proteins label the bacterial surface with ‘opsonins’, allowing more efficient phagocytosis. Neutrophils are the most important phagocytes, accounting for 60% of the leukocyte population in human blood. Indeed, complement C3 deficiencies and impaired neutrophil function are associated with

staphylococcal infections [4–6]. Furthermore, mice deficient in complement C5 are unable to clear *S. aureus* bloodstream infections [7]. *S. aureus* has evolved around 40 proteins with redundant functions in immune-response evasion, many of which target opsonization and neutrophils [8]. The majority of research into the interaction between antibodies, the complement system, and neutrophils is limited to planktonic staphylococci. However, both *S. aureus* and *S. epidermidis* are able to form biofilms on implanted devices (e.g., heart valves, catheters, prosthetic joints) and host tissues (e.g., chronic wounds, endocarditis, osteomyelitis) [9]. Biofilms are bacterial communities adhered to a biotic or abiotic surface with a self-made extracellular polymeric substance

## Abbreviations

AMPs, antimicrobial peptides; AP, alternative pathway; Aur, aureolysin; CHIPS, chemotaxis inhibitory protein of staphylococcus; CR, complement receptor; Eap, extracellular adherence protein; Ecb, extracellular complement-binding protein; Efb, extracellular fibrinogen-binding protein; EPS, extracellular polymeric substance; FLIPr/FLIPrL, formyl peptide receptor-like 1 inhibitor; Hla, alpha-hemolysin; Hlg, gamma-hemolysin; IgG, immunoglobulin G; LTA, lipoteichoic acid; LukAB, leukocidinAB; LukED, leukocidinED; Nuc, nuclease; PAMPs, pathogen-associated molecular patterns; PNAG, polymeric-N-acetyl-glucosamine; PRRs, pattern recognition receptors; PSMs, phenol soluble modulins; PVL, Pantón-Valentine leukocidin; ROS, reactive oxygen species; SAK, staphylokinase; Sbi, staphylococcal binder of IgG; SCIN, staphylococcal complement inhibitor; WTA, wall teichoic acid.

(EPS) that surrounds resident bacteria. Biofilm infections are difficult to treat because bacteria in a biofilm are unresponsive to antimicrobial therapy and are also protected from the adverse action of the immune system [9]. Furthermore, they often occur in bodily areas that cannot be easily treated. Consequently, medical treatments consist of long-term antibiotic regimens or removal of the infected implant. The increasing use of medical implants in the past 50 years has greatly contributed to the prevalence of implant-related infections [10]. Today, 25% of healthcare-associated infections are implant-related [11]. *Staphylococcus aureus* and coagulase-negative staphylococci cause 50–70% of orthopedic implant infections in Europe and the United States [12,13]. The low virulence of *S. epidermidis* compared with *S. aureus* explains why *S. epidermidis* biofilms are diagnosed only at a late stage, when there is a mature biofilm [14]. The most serious complication of biofilm-associated infection is the dispersal of biofilm-embedded bacteria, which can develop into sepsis. While *S. epidermidis* lacks most virulence factors expressed by *S. aureus*, these species are found with an almost identical frequency in implant-associated infections, suggesting that biofilm formation is also a very important evasion mechanism [12,14]. Hence, there is a need for innovative therapies that may counter staphylococcal biofilm infections, such as immune therapy. In order to develop such therapies, further insight into immune-evasion mechanisms of staphylococcal biofilms is needed, because these mechanisms may differ between planktonic and biofilm-growing modes [15].

In this review, we outline our current understanding of the role of antibody and complement opsonization in clearance of staphylococcal infections by neutrophils and how staphylococci escape from neutrophil effector functions. We include biofilm formation as an immune-evasion strategy and outline challenges in the development of therapies to treat established biofilm infections.

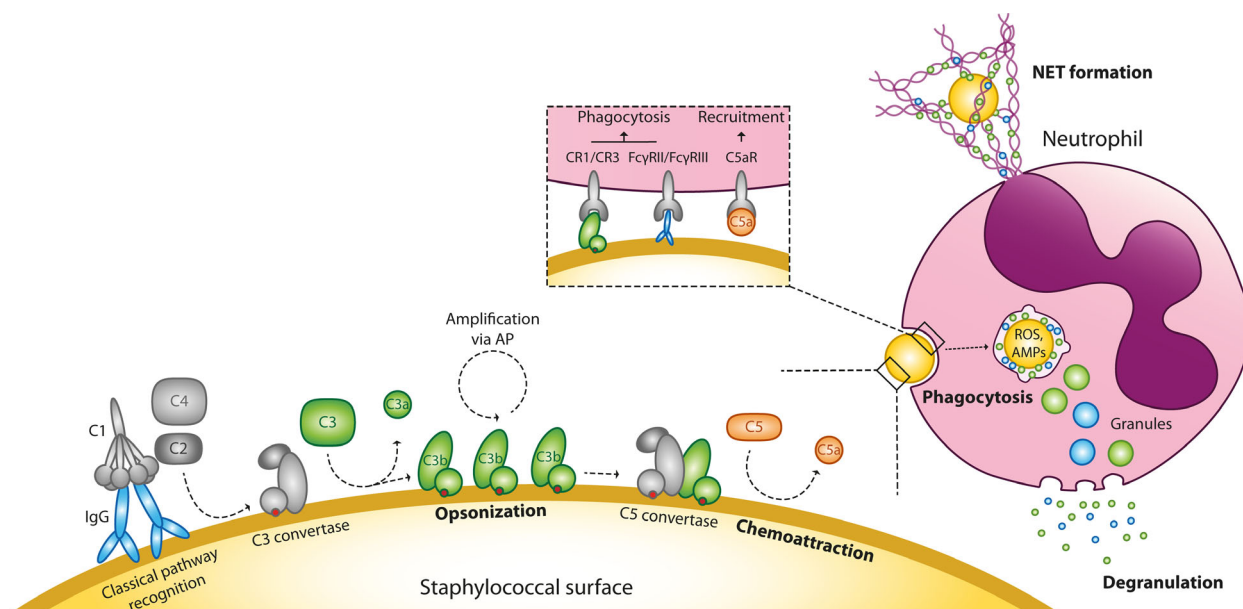
## The innate immune response against staphylococci

### Activation of the complement system

In staphylococcal infections, the primary role of the complement system is enhancement of binding and uptake by phagocytic immune cells *via* opsonization with C3b and iC3b and the attraction and activation of leukocytes through the release of anaphylatoxins C3a and C5a.

There are three pathways to activate the complement system: the classical pathway, the lectin pathway, and the alternative pathway (AP). Although complement is always present in the body, the molecular mechanisms driving the complement reaction at the bacterial surface are constantly changing within an individual. For instance, if a person has not been exposed to the bacterium before, complement is only activated by basic recognition pathways in which innate molecules (lectins, IgM by naive B cells) recognize conserved bacterial surface structures. The lectin pathway involves binding of mannose-binding lectins (MBLs) or ficolin and association of MBL-associated serine protease (MASP) complexes. MBLs bind wall teichoic acid (WTA) and peptidoglycan [16,17], and ficolins bind lipoteichoic acid (LTA) [18]. The AP lacks a specific recognition molecule and involves spontaneous breakdown of C3 which only occurs at surfaces that do not contain complement inhibitors, such as bacterial surfaces. The AP also functions as an amplification loop after C3b deposition on the bacterial surface *via* the lectin or classical pathways. When an individual develops specific antibodies against bacterial surface structures, these antibodies will induce the more specific classical complement pathway, which involves antibody binding and C1 deposition on the bacterial surface (Fig. 1) [19]. Naturally occurring antibodies in healthy humans have been found against various staphylococcal surface components, such as protein A, clumping factor A, and cell wall components such as WTA, peptidoglycan, and LTA [20–22].

All three complement activation pathways lead to the assembly of a C3 convertase on the staphylococcal surface. C3 convertases cleave C3 into C3a and C3b. C3a is released in the environment, and C3b becomes covalently linked to the staphylococcal surface and acts as an opsonin. Although all three pathways contribute to C3b deposition on staphylococcal surfaces, many studies indicate that the classical pathway is most important and required for optimal complement opsonization [20,23,24]. C3b can be processed to iC3b, which also acts as an opsonin but is not able to form AP C3 convertases and C5 convertases. Remaining C3b on the bacterial surface can form AP C3 convertases and thereby amplify the opsonization process. Later, high C3b densities on the surface lead to the change of C3 convertases into C5 convertases that cleave C5 into C5a and C5b [25]. C5a is a potent chemoattractant for phagocytes such as neutrophils [25]. The deposition of C5b on the bacterial surface leads to the formation of a lethal pore, the membrane attack complex, which kills Gram-negative bacteria



**Fig. 1.** The innate immune response against staphylococci. Recognition of staphylococci via the classical pathway involves IgG binding to the bacterial surface. Binding of C1 triggers the formation of C3 convertases that cleave C3 into C3a and C3b. The AP amplification loop leads to the accumulation of C3b on the bacterial surface. At high C3b densities, the C3 convertases switch substrate to C5. In turn, C5a recruits and primes neutrophils for phagocytosis, NET formation, or degranulation. Interaction of CR1/CR3 with C3b and FcγRII/FcγRIII with IgG leads to efficient phagocytosis. After phagocytosis, staphylococci are killed by high concentrations of AMPs and ROS.

within minutes. In contrast, Gram-positive bacteria such as staphylococci are resistant to direct killing by the membrane attack complex due to their thick peptidoglycan layer [26].

### Recognition by neutrophils

Neutrophils are the most important effector cells in staphylococcal infections. They are loaded with an arsenal of broadly effective antimicrobials, safely stored in granules which are only released upon activation. Neutrophils circulate in the bloodstream and are recruited to the tissue by chemoattractants that are locally produced following infection by the bacterium (e.g., formylated peptides [27]), the surrounding tissue (e.g., leukotriene B4 and platelet-activating factor), immune cells (e.g., IL-8 [28]), and activation of the complement system (e.g., C3a and C5a) [29]. Many of these molecules also act as neutrophil priming agents. Priming enhances the neutrophils' functional responses, such as phagocytosis, to a second stimulus [30]. Neutrophils become primed *via* formyl peptide receptors [31], C3a receptors (C3aR), and C5a receptors (C5aR) [32]. Furthermore, neutrophils get primed by recognition of pathogen-associated molecular patterns (PAMPs) with pattern recognition receptors (PRRs). PRRs include formylated peptide receptors

FPR1 and FPR2, Toll-like receptors, nucleotide-binding oligomerization domain-like receptors, and C-type lectin receptors. Staphylococcal PAMPs are mainly formylated peptides (recognized *via* FPR1 and FPR2), LTA (recognized C-type lectin receptors), peptidoglycan (recognized by NOD2), lipoproteins (recognized by TLR1/TLR2 and TLR2/TLR6 heterodimers), and CpG-DNA (recognized by intracellular TLR9) [33].

### Killing by neutrophils

Neutrophils recruited to the infection can kill bacteria by phagocytosis, degranulation of antimicrobial substances into the environment, or neutrophil extracellular trap (NET) formation (Fig. 1) [34]. The most effective way for neutrophils to eliminate staphylococci is intracellular killing in the phagolysosome after uptake. After phagocytosis, bacteria are subjected to high levels of reactive oxygen species (ROS) and degranulation of antimicrobial products into the phagosome [29]. Antimicrobial products effective against staphylococci present in the granules include lactoferrin, lysozyme, antimicrobial peptides (AMPs) such as LL-37, and neutrophil serine proteases [8,29]. In the extremely small volume of the phagosome, a very high local concentration of antimicrobial products and ROS can easily be reached, sufficient to create an

environment that is destructive to staphylococci [29,35]. Neutrophil granules can also fuse with the cytoplasmic membrane, releasing the contents into the surrounding tissue [34]. This way, the molecules can still have some antimicrobial functions, but they also cause tissue injury. A third killing mechanism is the formation of NETs. These are neutrophil DNA networks decorated with granule-derived AMPs that can entrap and kill microbes [36]. In the case of NETs, antimicrobial molecules are released into the environment, immediately trapped by the DNA-protein NET and thus present in a more 'concentrated' manner.

Phagocytosis is a very effective strategy of neutrophils to eliminate planktonic bacteria and even small aggregates of bacteria. Less is known about the neutrophil response against staphylococcal biofilms. Only a few studies indicate that neutrophils can bind, infiltrate, and phagocytose staphylococcal biofilms in the presence and absence of opsonins [37–42], but biofilms are more resistant to killing by neutrophils compared with planktonic bacteria [43]. The efficiency of phagocytosis seems to decrease as the biofilm matures [38]. Also, *S. epidermidis* biofilms are more resistant to killing than *S. aureus* biofilms [37]. Besides phagocytosis, neutrophils respond with ROS [41], degranulation [39–41], and NET formation [39–40,44,45]. The relative importance of different neutrophil effector mechanisms to biofilm clearance remains to be determined.

### Opsonization and phagocytosis

Opsonization is crucial for efficient phagocytosis of staphylococci by human neutrophils. Antibodies and the complement system both play an essential role in opsonization (Fig. 1). Complement receptors (CR) highly expressed by neutrophils are CR1 and CR3. CR1, a member of the family of complement proteins, recognizes opsonins C3b with high affinity and iC3b and C4b with lower affinity. CR3, a  $\beta$ 2-integrin, preferably binds to iC3b [25,46]. Immunoglobulin G (IgG) antibody opsonization by itself induces phagocytosis of staphylococci by neutrophils *via* interaction with Fc $\gamma$  receptors Fc $\gamma$ RII and Fc $\gamma$ RIII, but in presence of complement opsonization and subsequent engagement of CRs, this is strongly enhanced [47,48]. Phagocytosis is also enhanced through simultaneous priming by C5a and PAMPs [46].

The role of antibodies and the complement system in the neutrophil response against staphylococcal biofilms is not very clear. Most studies assessed the neutrophil response in the absence of opsonins [37–38,44,45], which is not very representative of the *in vivo* situation. Others use human serum as a source of opsonins but

do not study the role of opsonization in biofilm clearance by immune cells [39,42]. One study suggests that, comparable to the planktonic situation, the classical pathway seems most important for C3b deposition on *S. aureus* biofilms and this improves biofilm clearance by neutrophils [41]. However, this improved clearance was not mediated by phagocytosis but by enhanced ROS production [41]. Another study showed that on *S. epidermidis* biofilm, only the extracellular release of elastase was dependent on opsonization [40].

## Innate immune evasion by staphylococci

### Evasion by secreted proteins

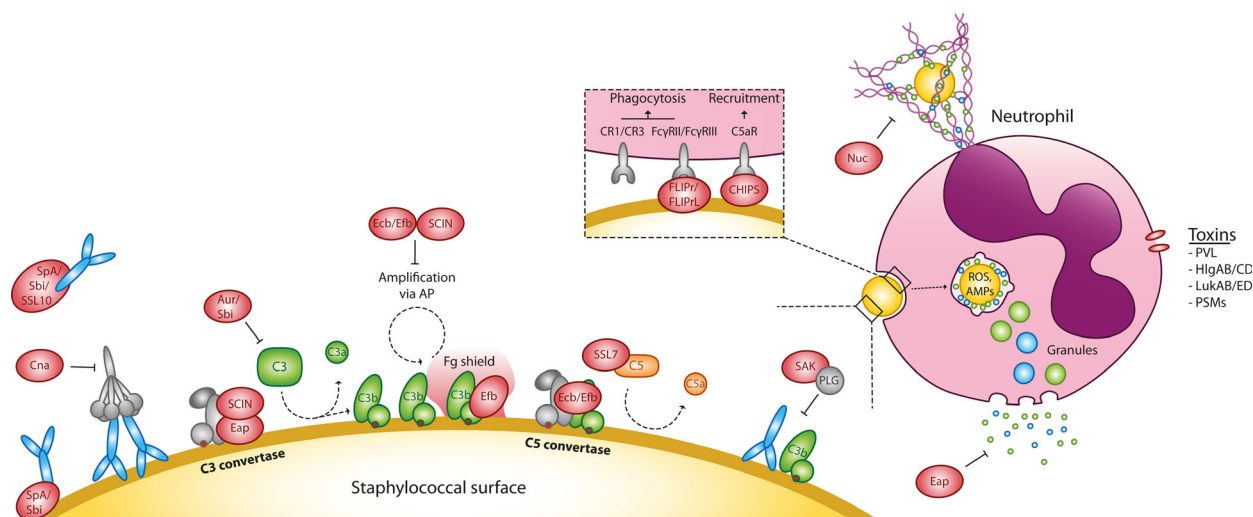
The importance of phagocytosis by neutrophils in the clearance of staphylococcal infections is further illustrated by the high number of evasion molecules that *S. aureus* has evolved against this process (Fig. 2). While other bacteria spend much energy on the production of a thick capsule to hide from immune recognition, the capsule formed by *S. aureus* does not completely block complement and antibody opsonization [49,50]. Instead, *S. aureus* is a specialist in immune evasion by secreted proteins.

### Evasion of opsonization and phagocytosis

First, the pathogen secretes several proteins to prevent antibody opsonization. Staphylococcal protein A and Staphylococcal binder of immunoglobulin (Sbi) bind IgG in the wrong orientation, thereby blocking Fc receptor-mediated phagocytosis [51,52]. Staphylococcal superantigen-like 10 binds IgG and not only inhibits opsonization but also inhibits activation of the classical pathway [53].

Second, *S. aureus* secretes multiple proteins that interfere with the complement system [8]. Collagen adhesin binds C1q and inhibits activation of the classical pathway [54]. Aureolysin (Aur) cleaves C3 in the surrounding of the bacterium, which is then degraded by host factors and not deposited on the bacterial surface [48]. Next to IgG binding, Sbi inhibits C3 cleavage into C3b by forming a complex with C3 and complement regulator factor H [55,56]. Extracellular adherence protein (Eap) blocks the formation of lectin and classical pathway C3 convertases [57] and Staphylococcal complement inhibitor (SCIN) binds to C3 convertases of the classical, lectin, and AP, thereby blocking C3b deposition and C5a production [47]. Extracellular fibrinogen-binding protein (Efb) and its homologue extracellular complement-binding protein





**Fig. 2.** Complement and neutrophil evasion by staphylococci. An overview of staphylococcal evasion molecules (red boxes) and their host targets.

(Ecb) block C3 and C5 convertases of the AP [58], thereby also lowering C3b opsonization and recruitment of neutrophils. Besides inhibition of convertases, Efb can also cross-link C3b to fibrinogen on the bacterial surface to build a 'capsule-like' shield to prevent recognition of opsonins by FcR or CR on neutrophils [59]. Another anti-opsonic protein is staphylokinase (SAK), which binds and activates plasminogen on the bacterial surface, which results in removal of opsonins IgG and C3b from the surface [60]. SSL7 binds to C5 and interferes with the production of C5a [61].

### Evasion of neutrophil recruitment and activation

Another group of secreted proteins evades priming and activation of neutrophils by blocking the interaction of chemoattractants with their neutrophil receptors [8]. For example, chemotaxis inhibitory protein of *Staphylococcus* (CHIPS) binds C5aR and FPR1 on neutrophils, thereby reducing recruitment toward C5a and formylated peptides [62,63]. Furthermore, FLIPr and FLIPr-like block FPR1 and FPR2 [64,65]. Moreover, FLIPr and FLIPr-like block multiple FcRs and inhibit antibody-mediated phagocytosis [66]. Other secreted proteins that interfere with neutrophil recruitment and priming are SSL3, SSL4, SSL5, SSL10, and staphopain A (ScpA) [8].

### Evasion of killing

*Staphylococcus aureus* has multiple strategies to survive in the different killing mechanisms of neutrophils. In short, these strategies include the secretion of nuclease

(Nuc) to escape from NETs, enzymes that protect from ROS, altering the charge of the cell wall envelope glycoproteins to increase tolerance to positively charged AMPs, and the secretion of neutrophil serine protease inhibitors such as Eap [8,67].

### Toxin production

Another class of secreted evasion proteins is toxins that kill immune cells directly by disrupting the cell membrane. The pore-forming toxins include alpha-hemolysin (Hla) and bicomponent leukocidins Pantone-Valentine leukocidin (PVL), gamma-hemolysin (HlgAB, HlgCB), leukocidinED (LukED), and leukocidinAB/GH (LukAB/GH). These toxins specifically target white blood cells *via* the interaction with receptors [68]. Hla does not lyse human granulocytes, thus neutrophils, but it does lyse other immune cells including lymphocytes and macrophages [69]. Staphylococcal PSMs are amphipathic structures that disrupt plasma membranes aspecifically. In serum, they are inactivated by lipoproteins. Because of this, they are only active in environments with very low to zero serum lipoprotein concentrations, such as in the phagosome [70]. While toxins are highly important contributors to *S. aureus* virulence, PSMs are the only virulence factors expressed by both *S. aureus* and *S. epidermidis* [2].

### Evasion by biofilm formation

*Staphylococcus aureus* and *S. epidermidis* are both known for their ability to form biofilms on implanted devices and host tissues. Especially for *S. epidermidis*,

which lacks most virulence factors that *S. aureus* expresses, biofilm formation can be seen as one of the primary immune-evasion mechanisms [14]. The staphylococcal biofilm EPS can consist of extracellular DNA, proteins, and polysaccharides. The composition is heterogenic between strains and species [9]. Most *S. aureus* isolates form protein-dependent biofilms [9], and *S. epidermidis* mostly forms polymeric-N-acetylglucosamine (PNAG)-dependent biofilms [71]. Probably for this reason, most research for biofilm-mediated immune evasion has been focusing on the role of PNAG in *S. epidermidis* biofilm and on the production of toxins by *S. aureus* biofilm. Below, we review our current knowledge about biofilm formation as an immune-evasion strategy (Fig. 3).

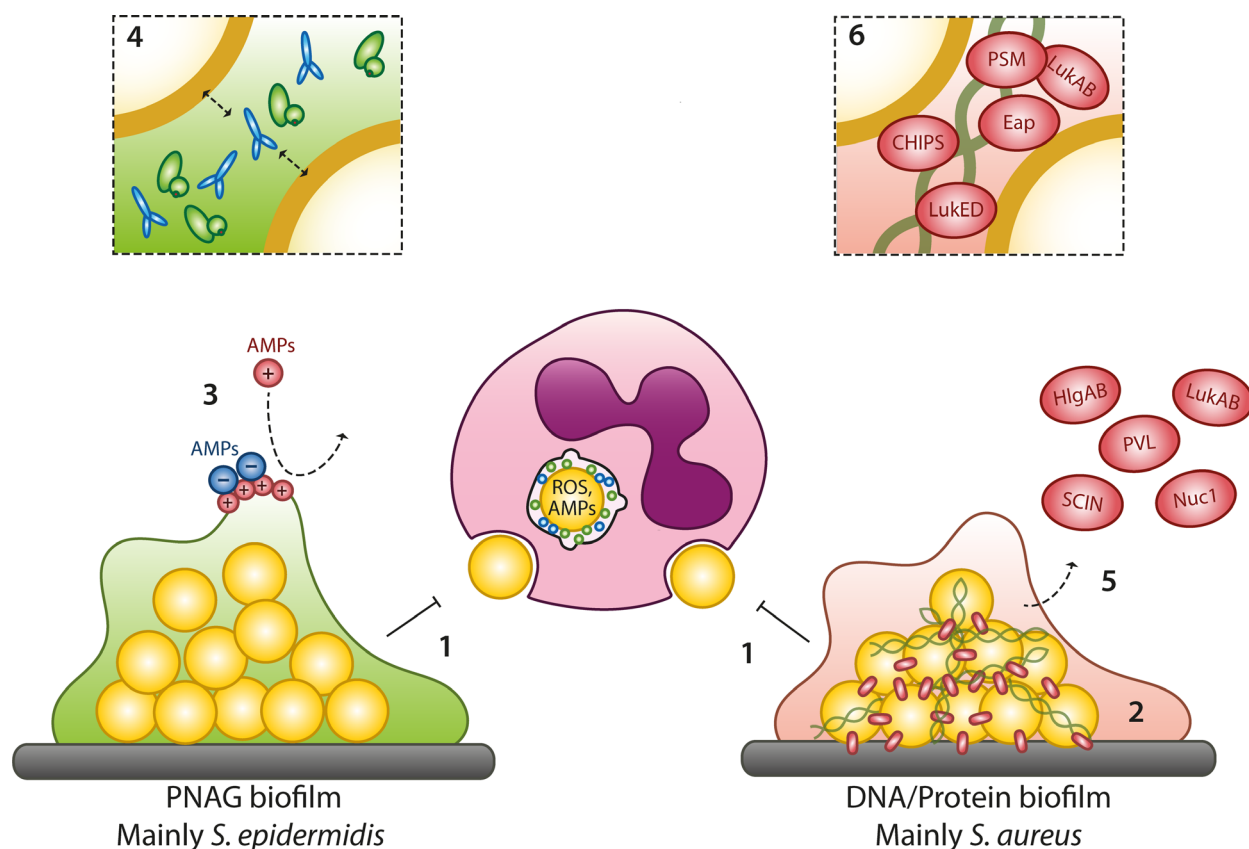
### Evasion by the EPS

Extracellular polymeric substance production is an important evasion mechanism in biofilms. EPS

components provide mechanic protection against phagocytosis, they protect from cationic AMPs, they may shield PAMPs present in the biofilm, and they are thought to act as a decoy for immune activation.

### Mechanical protection

Neutrophils are able to engulf particles up to their own cell size ( $\sim 10 \mu\text{m}$ ) [72]. When exposed to polystyrene beads bigger than  $11 \mu\text{m}$ , neutrophils exhibit frustrated phagocytosis. Thus, the ability of neutrophils to phagocytose bacterial aggregates bigger than  $10 \mu\text{m}$  depends on the ability of the phagocyte to break intercellular interactions and fragment the biofilm in smaller pieces. We know that phagocyte granules contain proteases (e.g., elastase, cathepsin G, and proteinase 3), DNases, and enzymes such as lysozyme that can hydrolyze peptidoglycan linkages [73,74]. This theoretically means that neutrophils are able to break down certain types of biofilm, but this remains to be



**Fig. 3.** Complement and neutrophil evasion by staphylococcal biofilms. Staphylococci can form PNAG- or eDNA/protein-dependent biofilms. The different types of biofilms employ distinct combinations of immune-evasion mechanisms: 1) EPS interactions mechanically protect from phagocytosis; 2) fibrin avoids immune recognition of PAMPs; 3) positive AMPs are repelled by PNAG, and negative AMPs are sequestered by PNAG; 4) EPS is opsonized instead of bacterial surface – EPS acts as a 'sink' for opsonization; 5) secretion of toxins and evasion molecules; and 6) incorporation of toxins and evasion proteins into the EPS.

confirmed. The presence of PNAG has been shown to protect *S. epidermidis* from phagocytosis by neutrophils [43]. In addition, both PNAG- and protein-dependent *S. epidermidis* biofilms are protected from phagocytosis by macrophages [75]. To our knowledge, neutrophils do not contain PNAG-degrading enzymes, which might explain the increased resistance to neutrophil phagocytosis of *S. epidermidis* biofilms compared with *S. aureus* biofilms [37].

#### Protection from AMPs

Most EPS components are negatively charged [76,77], but PNAG is highly positively charged [78]. On one hand, PNAG has been shown to protect against cationic host AMPs such as LL-37 by charge repulsion [79,80]. On the other hand, PNAG also protected against anionic AMPs, presumably by sequestering AMPs and keeping them away from the bacteria [80]. LL-37 was recently shown to have high antimicrobial activities against *S. aureus* biofilms, but it is unclear whether these biofilms were proteinaceous or polysaccharide-dependent [81].

#### Shielding from immune recognition

Next to providing mechanical protection, the EPS is thought to add to immune evasion by shielding PAMPs present on the bacterial surface. For example, *S. aureus* has been shown to incorporate fibrin in their EPS via coagulase expression, thereby protecting bacteria in a biofilm from immune recognition [82]. This would work similarly to capsule formation and Efb shielding, which are general mechanisms to shield bacteria from immune recognition (as described above). The same might apply to PNAG, but this has not been shown in literature [83].

#### Decoy for opsonization

The only staphylococcal EPS component that has been studied in the context of complement evasion by biofilms is PNAG. PNAG expression by planktonic *S. epidermidis* has been shown to reduce phagocytosis and killing *in vitro* and *in vivo*, when compared to their PNAG-negative isogenic mutants [43,79–80,84]. Interestingly, purified PNAG seems to be a very strong complement activator (measured by C3a and C5a release in supernatant) [83], but in the same study, PNAG-dependent *S. epidermidis* biofilms are paradoxically protected against IgG and C3b deposition and against phagocytosis and killing by neutrophils [43,83]. There can be different explanations for this protection

against biofilm opsonization. Kristian et al. suggest that when the complement system is highly activated on PNAG, it is not deposited close to the bacterial surface itself and therefore acts as a decoy for the actual target: the bacterium encased in the PNAG matrix. Another explanation could be that PNAG prevents the deposition of C3b on the EPS and after cleavage by the C3 convertase. The release of C3a does not necessarily mean that C3b has been deposited on the target. Despite diminished C3b deposition on the surface of the bacterium, high complement activation on PNAG could recruit phagocytes to the site of infection via C5a production, which could lead to better outcomes of the infection.

Besides a decoy for complement activation, PNAG could also act as a ‘sink’ for antibody opsonization. *In vitro* experiments revealed that IgG can penetrate biofilm, but antibodies against PNAG in rabbit serum rather bind to PNAG in the EPS than to PNAG on the bacterial surface [85]. Because of this, the biofilm was protected against antibody-mediated opsonic killing by neutrophils. Also, adding isolated PNAG to planktonic bacteria protected against phagocytic killing, indicating that an excess of PNAG in the biofilm captures antibodies and prevents binding close to the bacterial surface [85].

Even though antibodies, the complement system, and phagocytes are able to interact with staphylococcal biofilms, the right amount of opsonins and phagocytes close to the bacterial cell surface probably cannot be reached in a biofilm. The localization of antibody or complement opsonization in the biofilm (bacterial surface versus EPS) seems to be important for the immune response against bacteria in a biofilm. Opsonization of EPS does not necessarily lead to bacterial killing. Furthermore, the extracellular killing mechanisms of neutrophils could be directed to the EPS, and therefore, the effect at the bacterial surface could be limited and not sufficient to kill staphylococci.

#### Expression of immune-evasion proteins in biofilms

Recent proteomic and transcriptomic analyses showed that several evasion molecules (Eap, CHIPS, SAK, SSL10) and toxins (Hla, LukAB/GH, LukED, HlgAB, HlgCB, PSMs) are highly expressed in different *S. aureus* biofilm models compared with planktonic cultures of the same strain [76–77,86–88]. Most of these factors, especially Eap, were found to be incorporated into the EPS, while other factors are secreted into the environment as well [76,77]. Other factors including Sbi, Aur, SCIN, Ecb, Efb, FLIPr, FLIPrL, PVL, and Nuc are

also found in the EPS but were not highly expressed compared with planktonic *S. aureus* [76]. Recent publications propose that positively charged bacterial proteins interact with negatively charged eDNA and cell wall molecules in the *S. aureus* biofilm in order to maintain the biofilm structure [89]. Interestingly, all evasion proteins are positively charged and likely to be incorporated into the biofilm EPS network. For most of these factors, it is unknown whether they also maintain their evasion function when associated with the biofilm EPS.

Few studies have investigated the role of the above-mentioned proteins in biofilm immune evasion. For example, the secretion of SCIN and CHIPS was detected during early stages (from 3 h) of *S. aureus* biofilm formation [90]. The addition of recombinant SCIN to biofilm of a SCIN-negative strain decreased C5a release after incubation with human serum [90]. This indicates that *S. aureus* is able to evade the immune system already in early stages of the biofilm infection. In another study, the secretion of Nuc1 in early *S. aureus* biofilms was shown to protect against NET formation by neutrophils [45]. Furthermore, LukAB and Hla secretion by *S. aureus* biofilm induces cell death in macrophages [91]. Similarly, *S. aureus* biofilm induces cell death in neutrophils via the secretion of leukocidins PVL and HlgAB [44]. PSMs are known to play an important role in biofilm formation. They are produced in the end stages of biofilm formation under the strict control of *agr* [9]. Due to their amphipathic characteristics, PSMs disrupt noncovalent interactions between EPS molecules and they play a role in structuring and dispersal of *S. epidermidis* and *S. aureus* biofilms [9]. Here, they could also attack penetrating neutrophils because inhibiting serum lipoprotein concentrations might be low due to their large size (LDL ~ 500 kDa [70]). Whether this is true remains to be studied.

### Agr-dysfunctional isolates

The toxins described above are under *agr* control, and *S. aureus* biofilms have been shown to increase *agr* expression following neutrophil exposure *in vitro* [92]. Interestingly, Agr-dysfunctional *S. epidermidis* and *S. aureus* mutants are frequently isolated from biofilm-associated infections [93,94]. Because *agr* regulates dispersal via expression of proteases, Nucs, and PSMs [3], these mutants form dense and enlarged biofilms that are protected against neutrophil penetration and phagocytosis, despite the lack of *agr*-regulated toxin production [93,95]. The relative importance of evasion molecule secretion versus Agr dysfunctionality in

*S. aureus* biofilm immune evasion needs to be studied further. It remains unclear whether *S. aureus* biofilm is either completely Agr-functional or Agr-dysfunctional or whether these biofilms are composed of a mixed population [96]. There are studies indicating the Agr-positive population is not completely lost *in vivo* [96], so this could mean that part of the bacteria in the biofilm would protect the community by building a physical shield from the immune system and another part of the community will be actively releasing proteins to evade and attack the host immune system. This way, these biofilms will be extremely difficult to treat.

## Outlook

During staphylococcal infections, there is a continuous battle between staphylococci and innate immune system. In addition to counting on secreted factors to evade the immune system, staphylococci exploit the ability to form biofilms. Therefore, for such infections to be treated, we must increase our knowledge about biofilms and evasion of the innate immune response.

Most work on immune evasion has been based on planktonic bacteria. Planktonic cultures are very relevant for acute (bloodstream) infections, but less for chronic infections, the latter often involving biofilm formation. The field mainly relies on *in vitro* data and the question remains as to whether *in vitro* biofilms resemble those found *in vivo* [97]. *In vivo*, the formation of biofilms likely involves human factors and these bacterial communities are surrounded by the host tissue [98]. Moreover, there is a lack of appropriate animal models recapitulating biofilm formation in humans: Indeed, while biofilms can be exposed to the immune system of the patient for several decades, mice clear infections in < 8 days [98]. Future research should validate the findings described in this review using suitable *in vivo* models.

### Current research into antibiofilm strategies

Developing new treatments for established staphylococcal biofilm infections is challenging because biofilm composition and evasion strategies vary between species and even between isolates of the same species. Current research focuses on (a) the development of new antimicrobials that target bacteria in proteinaceous and polysaccharide-dependent biofilms [99]; (b) the use of biofilm-degrading enzymes, such as Dispersin B [10], DNases, or proteinases, to expose bacteria to antibiotics and to the immune system [11,102]; (c) targeting quorum-sensing networks; and (d) the use of therapeutic antibodies that neutralize evasion



molecules or boost the immune response [13]. The effect of all of these strategies is highly dependent on the species and biofilm EPS type. Most likely, successful therapies will be those that combine two or more of the above strategies. A major concern for biofilm-degrading enzymes is that they induce bacterial dispersal, which could result in acute infections if co-administered antibiotics or the immune system fail to clear circulating bacteria. Furthermore, regulatory quorum-sensing networks in staphylococcal biofilms are not completely understood and blocking networks such as Agr might turn to be counterproductive [93,95].

### Therapeutic antibodies for the treatment of staphylococcal biofilm infections

The natural immune response has more problems in clearing biofilm-related infections than infections by planktonic bacteria. However, neutrophils can recognize and phagocytose bacteria in a biofilm to a certain extent. As intracellular killing following phagocytosis is the most effective way for neutrophils to eliminate staphylococci, stimulation of this process seems a promising approach to combat biofilm infections. Antibodies are powerful players in the immune response because they opsonize bacteria thereby promoting phagocytosis both directly and indirectly, *via* their interaction with Fc receptors and the activation of the complement system, respectively. Scientific progress in the last decade has made it possible to produce human recombinant antibodies that can be administered as therapeutics in a safe and nonimmunogenic way [14]. Genetic engineering allows manipulating the effector function of these therapeutic antibodies or drug conjugation [15].

The first challenge in developing therapeutic antibodies for established biofilms is to determine the best target within the biofilm. Two studies have shown that rabbit IgG antibodies are able to penetrate *in vitro* biofilms [85,106], but the antibodies might be shielded from immune recognition *in vivo* by the biofilm EPS or host factors. Instead of being directed to the EPS, antibody binding and complement deposition need to be localized at the bacterial surface to ensure a localized response that is sufficiently strong to kill staphylococci. For *S. aureus* biofilms, targeting neutrophil-killing toxins with neutralizing antibodies might thus be necessary. A second challenge is that the ability of neutrophils to phagocytose bacteria from a biofilm depends on the ability to fragment the biofilm itself into smaller pieces. This might be ensured by coupling therapeutic antibodies with biofilm-degrading enzymes. A third challenge is that *S. aureus* survives into

phagolysosomes [70,107], where bacteria are protected from antibiotics and are able to spread to other sites in the body. To enhance intracellular killing of phagocytosed bacteria, therapeutic antibodies could be coupled to antibiotics that are activated upon phagocytosis [15].

Altogether, infection is a tug of war between the human immune responses and bacterial evasion strategies. To treat staphylococcal infections, these natural evasion mechanisms, including biofilm formation, need to be overcome to the advantage of the patients.

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