Trends in Microbiology

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Review

Fighting *Staphylococcus aureus* Biofilms with Monoclonal Antibodies

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***Staphylococcus aureus* (*S. aureus*) is a notorious pathogen and one of the most frequent causes of biofilm-related infections. The treatment of *S. aureus* biofilms is hampered by the ability of the biofilm structure to shield bacteria from antibiotics as well as the host's immune system. Therefore, new preventive and/or therapeutic interventions, including the use of antibody-based approaches, are urgently required. In this review, we describe the mechanisms by which anti-*S. aureus* antibodies can help in combating biofilms, including an up-to-date overview of monoclonal antibodies currently in clinical trials. Moreover, we highlight ongoing efforts in passive vaccination against *S. aureus* biofilm infections, with special emphasis on promising targets, and finally indicate the direction into which future research could be heading.**

Highlights

S. aureus and other staphylococci are the most common causes of persistent biofilm-associated infections, which are inherently resistant to antibiotics as well as the host's immune system.

Antibody-based approaches can be used to combat biofilms. Antibodies can prevent bacterial attachment and/or biofilm maturation, or even disperse mature biofilms, as shown in *in vitro* and preclinical studies.

Several sophisticated techniques can be used for the generation of human monoclonal antibodies, to be ultimately employed in research or clinical settings.

Since antibodies against surface structures proved unsuccessful in clinical trials so far, current research is focussed on *S. aureus* toxins, and biofilm matrix components.

Multivalent vaccines, with a special emphasis on biofilm-related targets, are the strategy of choice for active as well as passive vaccination.

Clinical Significance of *S. aureus* Biofilm-associated Infections

The Gram-positive pathobiont *S. aureus* is one of the most frequent causes of nosocomial infections; however, no vaccine is yet available. *S. aureus* infections are highly diverse, ranging from acute diseases, such as bacteremia and skin abscesses, to severe chronic infections that are often associated with biofilms [1]. Due to an arsenal of **adhesins** (see Glossary), *S. aureus* can attach to and persist on host tissues (e.g., heart valves, and bones) as well as implanted materials (e.g., catheters, prosthetic joints, and pacemakers), and cause diseases such as endocarditis, and osteomyelitis [1–3]. On the other hand, about 20% of the human population is persistently colonized in the anterior nares and other body sites such as the intestine, while the remainder carry the bacteria intermittently [4]. In most cases, colonization is asymptomatic, but it can also lead to endogenous infections [5].

Over the past decades, the steady increase in the use of medical implants has been accompanied by a rise in infection risk. Indeed, implant- or device-associated infections are important complications associated with the use of biomaterials [2,6], and they account for one quarter of all healthcare-associated infections in the USA [7]. Among their deleterious consequences are failure of prosthetic devices, implant replacement with its associated risk of clinical complications, and chronic and/or relapsing diseases [2,8]. Staphylococci, including *S. aureus*, *Staphylococcus epidermidis* (Box 1) and other coagulase-negative staphylococci (CoNS) are the main culprits of foreign body-associated infections, accounting together for an estimated 80% of all infections [2,9]. The diagnosis and targeted therapy of implant-associated infections is often problematic, because they are frequently subclinical, and culture-negative.

Biofilm formation is an important virulence mechanism of many bacterial pathogens. A biofilm is defined as a sessile microbial community embedded within an amorphous slimy material [2]. Biofilm formation enables growth on natural and foreign surfaces, and it shields bacteria from antibacterial therapies as well as the host's immune system, often leading to persistent infections unresponsive to antibiotic therapy [2]. In addition to the matrix representing a

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Box 1. Antibody-based Therapies against *S. epidermidis* Biofilm-associated Infections

S. epidermidis is the most frequent cause of device-related infections, with biofilm formation as the major virulence factor [2,9,121]. Comparable to *S. aureus*, targeting *S. epidermidis* biofilm-associated infections can be achieved either by preventing bacterial attachment to implants, or by blocking cell-to-cell adhesion during biofilm maturation. However, unlike *S. aureus*, *S. epidermidis* biofilm formation relies mainly on exopolysaccharides rather than proteins [20,122].

The two major biofilm matrix constituents, PNAG and Aap, have been targeted by monoclonal antibodies in order to prevent biofilm formation. Anti-PNAG antibodies inhibited biofilm formation *in vitro* and were protective in a rabbit endocarditis model [123]. However, the inhibitory effect on static biofilm formation seems to be strain-dependent [124]. Apparently PNAG, as a biofilm matrix constituent, hinders antibody binding close to the bacterial cell surface, which is needed for efficient opsonic killing [45]. The surface protein Aap promotes cell-to-cell adhesion within a biofilm. Monoclonal antibodies against Aap reduced *S. epidermidis* biofilm formation *in vitro*, but neither enhanced opsonophagocytosis nor protected mice in an experimental biomaterial-associated infection [110,125]. The lack of protection might result from shedded Aap, acting as a decoy for anti-Aap antibodies [125].

The anti-LTA monoclonal antibody Pagibaximab was designed primarily for the treatment of *S. epidermidis* biofilm-associated sepsis, which occurs particularly often in neonates [126,127]. After encouraging results in animals, and a more limited phase II study in very low birth weight neonates, a larger phase III study in a similar patient cohort failed to show a reduction in staphylococcal sepsis (Table 2) [108].

As there are no toxins in *S. epidermidis* other than PSMs [128], antitoxin monoclonal antibody development for the treatment of *S. epidermidis* catheter-related bacteremia has been limited to those peptides. This approach is, however, problematic due to the diversity and high production of PSMs. Although anti-PSMβ polyclonal antibodies showed some success in limiting the dissemination of *S. epidermidis* biofilm-associated infection in mice [33], an octavalent antigen mixture containing four α-type PSMs, despite their immunogenicity, did not protect against *S. aureus* bacteremia [129], dampening the enthusiasm for passive PSM-targeted vaccination approaches.

A more suitable candidate might be the surface protein SesC, which is expressed in biofilm-associated as well as planktonic cells. Polyclonal rabbit sera against SesC partially prevented *in vitro* biofilm formation by *S. epidermidis* and dissolved established biofilms [130]. A similar reduction in biofilm formation was observed with polyclonal anti-SesC antibodies in a mouse model of catheter-related infections [131].

penetration barrier for many antimicrobial agents, the efficacy of most antibiotics is reduced against biofilms, because cells in a biofilm are in a state of reduced metabolism [10,11], whereas most antibiotics target active cell processes, such as cell wall formation, translation, or transcription [12]. Consequently, there is an immense medical need to develop innovative preventive and/or therapeutic interventions, including anti-infective biomaterials, biofilm-active antibiotics, and biofilm matrix-degrading enzymes [13]. Another appealing measure to prevent biofilm formation and/or treat established biofilms is the use of **monoclonal antibodies** (**mAbs**) targeting the invasive pathogen, which is the focus of this review. After describing aspects of *S. aureus* biofilm formation, the antibacterial **antibody** response in *S. aureus* biofilm infections, and techniques for generating monoclonal antibodies, we provide an update on preclinical as well as clinical studies on monoclonal antibodies against *S. aureus* (biofilm-associated) infections, and outline critical aspects for the development of a successful anti-biofilm vaccine.

Staphylococcal Biofilm Stages and Composition

In order to develop protective antibody-based therapies, it is essential to gain an in-depth understanding of both the process of biofilm formation, and the composition of biofilms, since gene and protein expression differ greatly between the planktonic and biofilm modes of bacterial growth [14,15]. In the past, proteomic analyses were usually based on examining intracellular proteomes of laboratory isolates in static biofilms. However, more recent studies used flow chamber systems or even analyzed biofilms from animal infection models, which may reflect the human clinical situation more closely, and hence reveal novel biofilm-associated targets [14,16–19].

Glossary

Active vaccination: immunization with an antigen to provoke adaptive immunity. It usually induces long-lasting and robust protective immune memory, but requires several weeks to become fully effective.
Adhesins: bacterial cell surface proteins that enable bacteria to bind to the surfaces of host cells or to components of the extracellular matrix.
Antibody: also known as immunoglobulin (Ig), is secreted by B cells upon antigen contact. It is highly specific and binds its target structure, the antigen, with very high affinity. While each B cell produces identical antibodies, an individual can produce a total number of at least 10^7 antibody specificities, enabling the immune system to respond to a wide range of antigens. Structurally, antibodies are heterodimeric proteins composed of two heavy and two light chains, which are linked by disulfide bonds.
Chimeric antibodies: antibodies whose constituent parts are derived from different species, mostly human and murine. The replacement of the murine with a human Fc part allows chimeric antibodies to efficiently interact with the human immune system, and reduces the risk of an adverse immune response to the applied monoclonal antibodies.
Human antibody: antibody that is composed of fully human antibody heavy and light chains.
Humanized antibody: an antibody in which mouse antigen-binding regions (= hypervariable loops) are genetically engineered into otherwise human antibodies.
Hybridoma: hybrid cell line formed by fusing a myeloma cell (no antibody production, but immortal) with a specific antibody-producing B cell (antibody production, but mortal). The resulting immortal hybridoma cells are grown in tissue culture and produce antibodies of a single specificity (i.e., monoclonal).
Monoclonal antibodies (mAbs): antibodies produced by a single clone of B cells, which are hence all identical. They are generated either by immortalizing the antibody-producing B cell or by cloning the respective genes into an expression system.

Biofilm formation in staphylococci has been described as a process comprising at least three main stages: (i) bacterial attachment to a surface, (ii) biofilm formation and maturation, and (iii) biofilm detachment/dispersal (Figure 1, Key Figure) [20]. Staphylococcal agglomerations that are not attached to a surface are also occasionally regarded as biofilms [21]; in those cases, intercellular aggregation substitutes for the initial adhesion step.

Attachment of bacteria to abiotic plastic surfaces of indwelling medical devices may happen via hydrophobic attraction. However, soon after insertion, human matrix proteins cover the device surfaces, and thus, initial attachment *in vivo* proceeds mainly via the interaction of staphylococcal surface-binding proteins with human extracellular matrix [20]. Many of the former belong to the **'microbial surface components recognizing adhesive matrix molecules' (MSCRAMMs)** family [22]. MSCRAMMs (discussed in detail in the section headed 'Preclinical Studies on Antibodies Targeting *S. aureus* Biofilms') are anchored to the cell wall via the enzyme sortase, and contain cell wall-spanning domains that end with an exposed domain binding to human matrix proteins (Figure 1). Overall, there is a pronounced redundancy among the MSCRAMMs, reflecting their key role in bacterial colonization and survival in the host [22].

The biofilm formation/maturation phase, in addition to bacterial growth, is characterized by the secretion of biofilm matrix components and the creation of a three-dimensional biofilm structure. The composition of the biofilm matrix is heterogeneous, comprising proteins, extracellular DNA (eDNA) and polysaccharides (discussed in detail under 'Preclinical Studies on Antibodies Targeting *S. aureus* Biofilms'). Several secreted proteins have been implicated in biofilm formation; many of them are surface-binding proteins, whose contribution to the initial adhesion versus subsequent phases of biofilm development is often hard to discern. In contrast, the *S. epidermidis* accumulation-associated protein Aap, and its *S. aureus* homologue SasG, appear to have a very specific biofilm matrix function, forming polymeric fibrils that link together cells in a biofilm [23–25].

Another biofilm-characteristic component is the cell surface-associated exopolysaccharide PNAG (poly-*N*-acetyl-β-(1,6)-glucosamine, also called polysaccharide intercellular adhesin, PIA) [26]. PNAG is not omnipresent in staphylococcal biofilm-forming isolates [27], but its production supports cell-to-cell adhesion, leading to more robust biofilms [28,29]. PNAG's cationic nature facilitates bacterial attachment to host cell surfaces [30], which is possibly mediated by negatively-charged molecules such as teichoic acids and eDNA, which is released by dying cells.

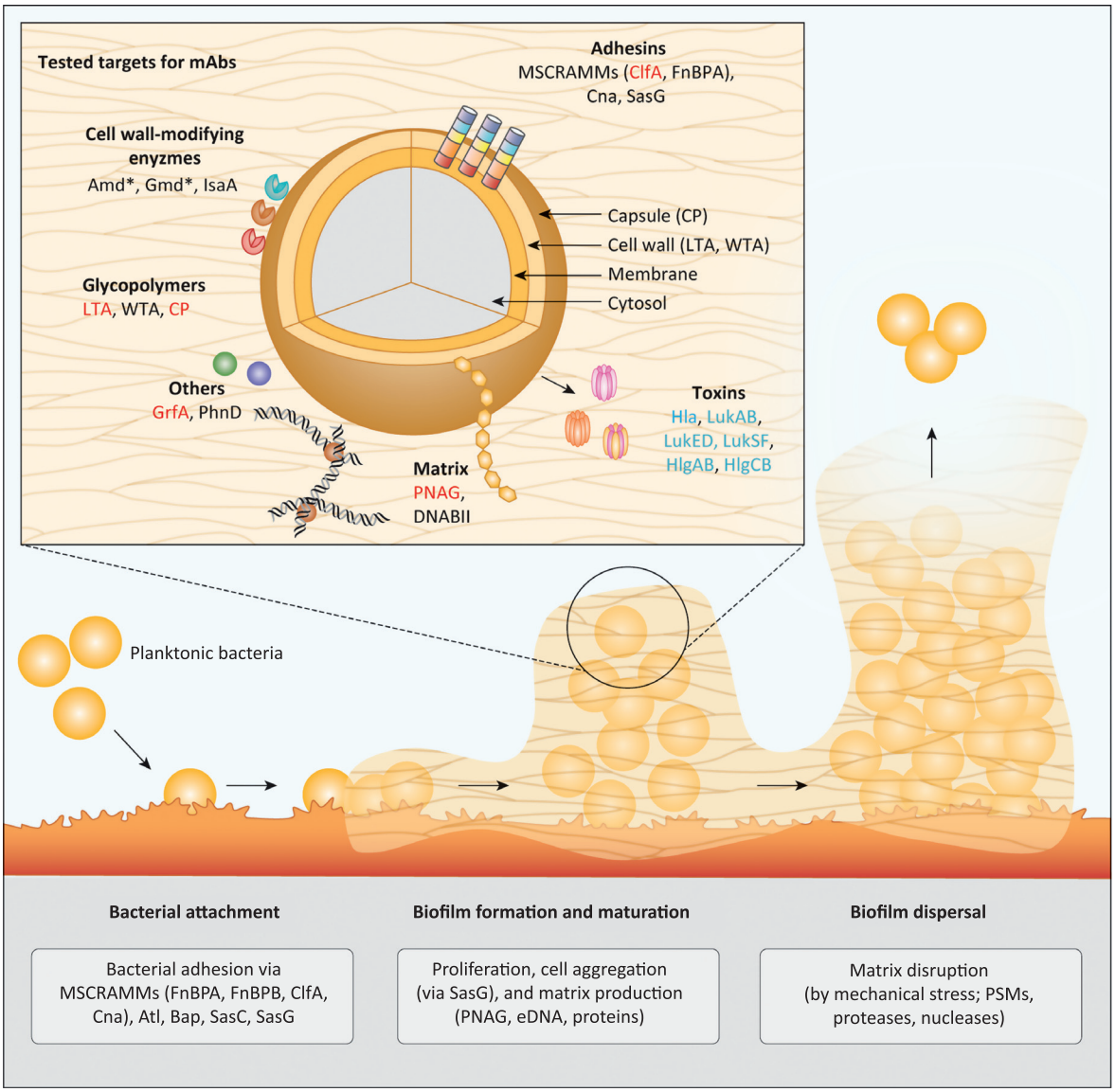
Biofilms do not grow as undifferentiated 'bricks', but contain channels that are deemed important for nutrient delivery to all layers of a biofilm. Enzymatic digestion of biofilm matrix molecules, such as eDNA and proteins, by nucleases and proteases, respectively, has been implicated in channel formation [1]. However, no enzyme capable of degrading PNAG has so far been identified in staphylococci. Moreover, phenol-soluble modulins (PSMs) are amphipathic and surfactant-like peptides that structure biofilms independently of biofilm matrix composition, most likely by disrupting hydrophobic as well as hydrophilic interactions between biofilm matrix molecules [31,32].

Detachment of cells or cell clusters from a biofilm can be triggered solely by mechanical shear forces, as encountered in the blood stream. However, this process, which is crucial for the systemic dissemination of a biofilm-associated infection, can also be facilitated by pronounced activity of biofilm-structuring factors. For instance, PSMs disrupt interactions of biofilm matrix molecules, such as PNAG, with each other *in vivo*, contributing to biofilm dispersal [31,33].

Membrane attack complex (MAC): a protein complex, composed of the terminal complement proteins, which generates lytic pores in certain pathogens.
Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs): cell wall-attached adhesin proteins, which share a similar protein structure and a common mechanism of ligand binding; they include ClfA, ClfB, SdrC, SdrD, SdrE, bone sialoprotein-binding protein, FnBPA, FnBPB, and Cna. They mediate the initial attachment of bacteria to abiotic/biotic surfaces, providing a critical step in the establishment and persistence of infections.
Murine antibodies: antibodies that have been generated in mice. They are recognized by the human immune system as foreign antigens, and can thus – upon repeated application – lead to allergic reactions, reduced therapeutic effectiveness, and shorter circulating antibody half-life.
Opsonophagocytic killing (OPK): the deposition of antibody and/or complement onto the surface of a pathogen, making it more easily ingested by phagocytes.
Passive vaccination: transfer of antibodies/immune sera/immune cells to a host, in order to provide immediate and specific – albeit short-lived – immunological protection.

Key Figure

Overview on Tested Targets for an Antibody-based Preventive or Therapeutic Strategy against Biofilm-associated *Staphylococcus aureus* Infections



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Antibacterial Antibody Response in *S. aureus* Biofilm Infections

How the innate and adaptive immune systems react to biofilms, and what type of immune response is protective, is still not well understood. Biofilm infections trigger an inflammatory response, as reflected by the infiltration and activation of phagocytes at the site of infection, the release of proinflammatory cytokines, promoting a Th1/Th17 response, and the production of antibodies, predominantly of the human IgG1 subclass [34–36]. While neutrophils are capable of infiltrating the biofilm, and are able to phagocytose enclosed cells efficiently, this defense mechanism is less effective in mature biofilms [37]. This exemplifies the inefficiency of the induced host response in clearing a persistent biofilm infection [37,38].

Similarly, the protective potential of antibodies in biofilm infections is not well defined. *S. aureus* infection stimulates the production of specific antibodies against a broad range of surface and secreted staphylococcal proteins, but these generally do not prevent a reinfection with this notorious pathogen [39]. However, antibody profiling in sepsis patients at the time of diagnosis showed that high antibody titers might confer protection from an adverse outcome [40]. This implies that the immunological 'starting position' is important for disease outcome, a fact that is encouraging for efforts in vaccine development. For biofilm infections, clinical data are scarce, but they suggest that biofilms also trigger or boost an antibody response against a broad range of *S. aureus* antigens: adhesins and cell wall-modifying enzymes, biofilm matrix components, toxins, and immune evasion factors (discussed in detail under 'Preclinical Studies on Antibodies Targeting *S. aureus* Biofilms') [41–43].

In line with these patient data, animal experiments indicate that boosting the antibody response by **active** or **passive vaccination** prevents, or at least reduces, the severity of biofilm-associated *S. aureus* infections [18,35,44]. For example, in a murine model of mesh-associated biofilm infection, a vaccination approach using biofilm matrix exoproteins significantly reduced the number of bacterial cells inside a biofilm and on the surrounding tissue [18]. Another multivalent *S. aureus* vaccine, comprising four cell wall-associated proteins, prevented the formation of biofilm-mediated osteomyelitis in the majority of the treated animals when combined with an antimicrobial therapy [44]. Hence, animal data suggest that antibodies can contribute to biofilm prevention and clearance.

Anti-*S. aureus* antibodies can penetrate the biofilm matrix [45,46] and interfere with all three stages of biofilm formation. Initial attachment can be prevented by targeting surface-bound or soluble adhesins (Figure 2). Biofilm maturation is disturbed by blocking surface proteins

Figure 1. *Main figure.* Biofilm formation in staphylococci comprising three main stages: bacterial attachment to a surface, biofilm formation and maturation, and biofilm detachment/dispersal. For the attachment to (a)biotic surfaces, *S. aureus* relies on a broad spectrum of functionally redundant adhesins such as the MSCRAMMs (ClfA, Cna, FnBPA, FnBPB). After successful adhesion, bacteria start proliferation and production of the biofilm matrix, consisting of eDNA (stabilized by DNABII), PNAG, and proteins. Eventually, biofilm dispersal is mediated by mechanical shear stress (e.g., in a blood vessel) or by dispersion factors like PSMs, nucleases, and proteases. *Insert*. Molecular targets for antibody-based therapies tested in preclinical and in clinical studies include adhesins and cell wall-modifying enzymes, other cell wall-attached proteins, surface glycopolymers, biofilm matrix components, as well as toxins and immune evasion proteins. Targets from preclinical studies, ongoing clinical trials, and failed clinical trials are shown in black, blue, and red, respectively. The asterisk indicates that the *S. aureus* protein autolysin (Atl) is proteolytically processed into two enzymes, autolysin amidase (Amd) and autolysin glucosaminidase (Gmd), which stay non-covalently attached to the cell surface.
Abbreviations: Atl, autolysin; Amd, autolysin amidase; Bap, biofilm-associated protein; ClfA, clumping factor A; Cna, collagen-binding protein; CP, capsular polysaccharides; DNABII, DNABII family proteins; eDNA, extracellular DNA; FnBPA/FnBPB, fibronectin-binding protein A and B; Gmd, autolysin glucosaminidase; GrfA, ABC transporter; Hla, α-toxin; Hlg, γ-hemolysin; IsaA, immunodominant staphylococcal antigen A; LTA, lipoteichoic acid; Luk, leukotoxins; mAb, monoclonal antibody; MSCRAMMs, microbial surface components recognizing adhesive matrix molecules; PhnD, subunit of alkylphosphonate ABC transporter; PNAG, poly-*N*-acetyl-β-(1,6)-glucosamine; PSMs, phenol-soluble modulins; SasC/G, *S. aureus* surface protein C and G; WTA, wall teichoic acid.

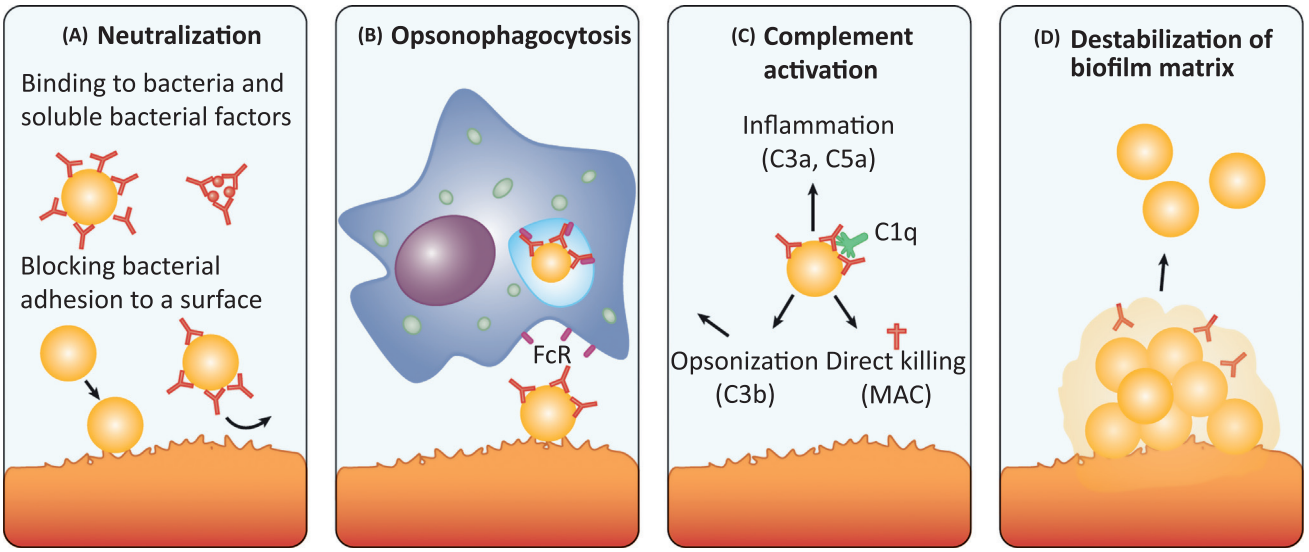
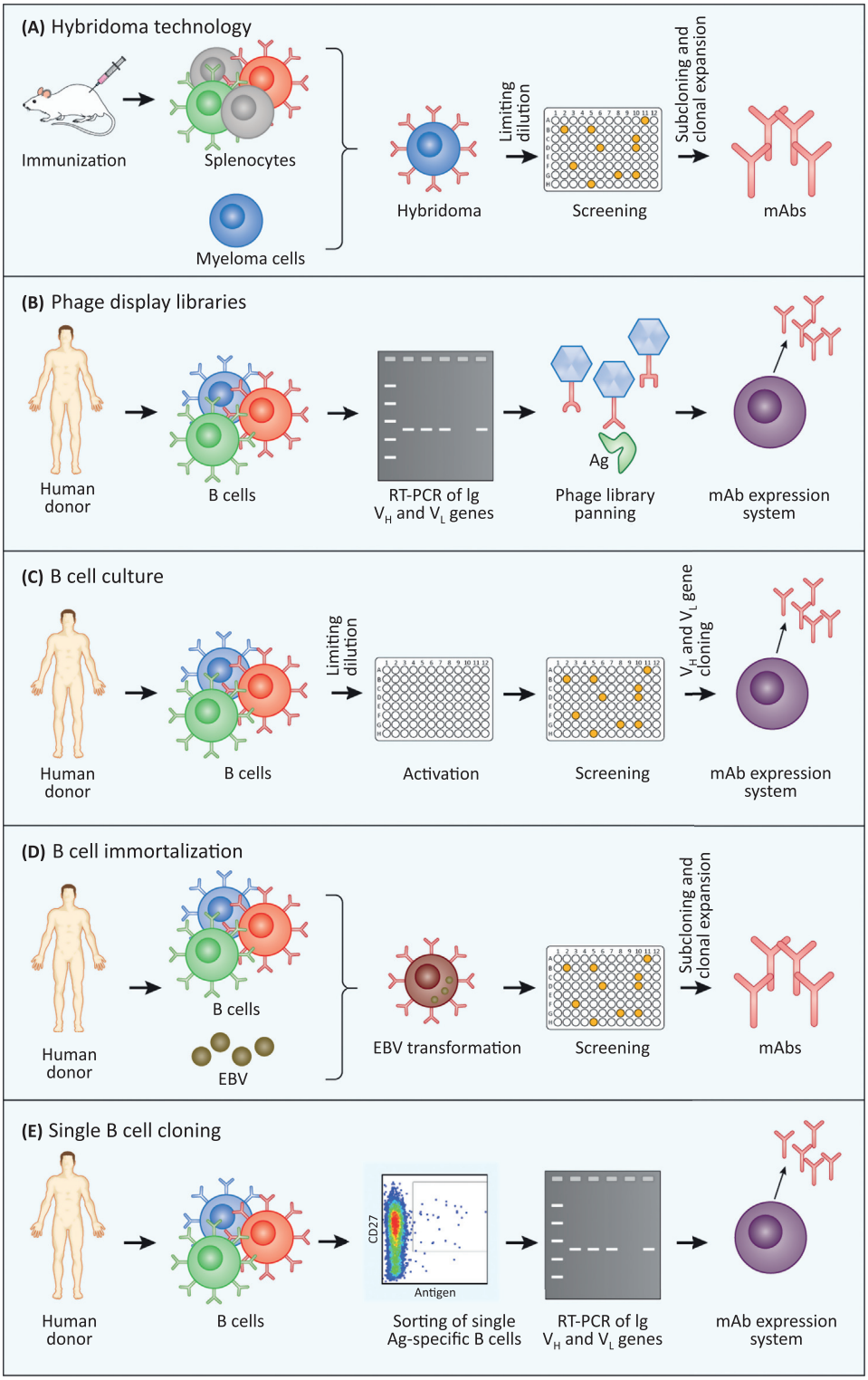


Figure 2. Antibodies Can Interfere with Biofilm Formation and Promote Dispersal of Established Biofilms by Several Mechanisms. (A) Secreted staphlyococcoal proteins (e.g., immune evasion molecules, toxins, exoenzymes), as well as surface proteins, are involved in biofilm development and are hence potential targets for therapeutic purposes. High-affinity IgA and IgG antibodies can neutralize the action of bacterial toxins and surface proteins. Moreover, antibodies can bind to bacterial adhesins (e.g., ClfA, FnBPA) and cell wall components (e.g., PNAG), thereby blocking initial attachment to host matrices and subsequent initiation of biofilm formation. (B) Surface-bound antibodies (most prominently IgG) can trigger bacterial uptake and destruction by neutrophils and macrophages expressing Fc receptors (FcR) on their surface (opsonophagocytosis). Activation of neutrophils can also trigger granule release, oxidative burst, and NETosis. (C) Surface-bound antibodies (IgM and IgG) trigger complement activation via the classical pathway. Following binding of C1q to the surface-bound antibody, the complement cascade is initiated, resulting in the formation of the C3 convertase, which cleaves the central component of all complement pathways, C3, into C3a and C3b. C3b acts as an opsonin, enabling phagocytes that express the C3b receptor to ingest C3b-coated bacteria more easily. The soluble C3a (as well as C5a) act as chemoattractants that recruit immune cells to the site of infection, causing inflammation. C3 activation also triggers the formation of the **membrane attack complex** (**MAC**) that generates lytic pores in certain pathogens. Gram-positive bacteria, including *S. aureus*, are protected from MAC-dependent lysis by their thick peptidoglycan layer [132]. (D) Antibodies targeting different components of the biofilm matrix, for example DNABII, can destabilize a biofilm matrix and thereby promote bacterial dispersal and clearance by immune cells or antibiotics.
Abbreviations: FcR, Fc receptor; MAC, membrane attack complex.

involved in cell-to-cell adhesion, and biofilm dispersal is enhanced by targeting matrix-stabilizing proteins. Moreover, high-affinity IgA and IgG antibodies can neutralize secreted bacterial factors (e.g., toxins, enzymes, and immune evasion molecules). Finally, surface-bound antibodies can enhance biofilm elimination by neutrophils and macrophages, either via antibody-binding Fc receptors or by inducing complement activation and C3b deposition on the bacterial surface (Figure 2) [18,47,48]. In conclusion, antibodies can potentially interfere with biofilm formation and/or promote dispersal of established biofilms by several mechanisms. However, since the natural antibody response in many cases seems to be insufficient to eliminate established biofilms, boosting the antibody response by active or passive vaccination seems a promising approach to reduce the severity of biofilm-associated *S. aureus* infections.

Generation of Monoclonal Antibodies

Monoclonal antibodies are superior to polyclonal sera in studying anti-biofilm activities, since they allow for molecular interaction studies, and can potentially be applied in human patients. Over the past decade, technical advances have been made in the production and modification of monoclonal antibodies. Traditionally, monoclonal antibodies were generated using the



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hybridoma technology (Figure 3A), resulting in **murine antibodies,** which can, however, have severe side effects if introduced into the human host [49,50].

Within less than a decade after the first monoclonal antibody was described, two approaches were developed to reduce antigenicity and enhance antibody-mediated effector functions. The recombinant attachment of a murine antigen-specific variable region to a human constant region resulted in the rise of **chimeric antibodies** [51]. Almost concurrently, the first **humanized antibody** was generated by transferring the murine complementarity-determining regions (CDRs) into a **human antibody** sequence. Especially the latter approach reduced the murine proportion, and hence the immunogenicity of the antibody [52]. Both methods enabled the selection of the desired human constant region, which defines the antibody class and hence antibody-mediated effector functions. Nowadays it is possible to generate chimeric and humanized antibodies by combining the use of transgenic mice expressing chimeric or CDR-drafted antibodies with the hybridoma technology.

The four most common techniques for the generation of numerous fully human monoclonal antibodies are phage display libraries, B cell cultures, B cell immortalization using Epstein–Barr virus (EBV), and cloning antibodies from single antigen-specific B cells (Figure 3B–E). While phage display libraries are generated by random combination of immunoglobulin heavy and light chains, the other three methods are based on antigen-specific B cells from exposed donors and thus reflect the physiological antibody response [53,54]. These methods can also be elegantly combined. For example, unstable hybridomas and EBV-transformed cell lines can be rescued by cloning the antibody-coding sequences into an expression system.

Using antibody engineering, it is also possible to tailor the affinity and effector functions of antibodies to their application. For example, monoclonal antibodies can be genetically modified to increase antigen affinity and antibody half-life or to increase/decrease affinity towards Fc receptors [55,56]. Moreover, two antibodies can be combined to create bispecific antibodies

Figure 3. Methods Commonly Used for the Generation of Monoclonal Antibodies (mAbs). (A) Hybridoma technology. Following immunization with an antigen, mice start producing large amounts of antigen-specific B cells. These cells are harvested from the spleen and fused with myeloma cells. The resulting hybridoma cells are screened for the secretion of antigen-specific antibodies. Antigen-specific hybridoma cells are selected by limiting dilution (subcloning) [3]. (B) Phage display library. Initially, mRNA is isolated from B cells or plasma cells and then reverse-transcribed into cDNA. The variable light and heavy chains are amplified via PCR and ligated into a phage display vector. The resulting phage library consists of 10^8–10^{10} different phages, each encoding a single surface-expressed mAb, generated by random combination of heavy and light chains. The antigen is subsequently 'displayed' to the phage library in successive rounds, to enrich antigen-specific phages (panning). The genes encoding the desired antigen-specific mAbs can then be cloned into an appropriate expression system for the generation of the mAbs of interest [1,2]. (C) B cell culture. After isolation and limiting dilution, B cells are cultivated and activated *in vitro*, leading to the secretion of antibodies. B cell culture supernatants are screened for antigen-specific antibodies, and positive cultures are used for the amplification of heavy and light Ig genes via PCR. The antibody sequence is finally cloned into an expression system to produce the mAbs [6]. (D) EBV immortalization. Human B cells or plasma cells are isolated and immortalized using Epstein–Barr virus, followed by single cell distribution. Supernatants of B cell cultures are screened for specific antigen-binding and subsequently subcloned to produce mAbs [4]. (E) Single B cell cloning. Human B cells are isolated, and single antigen-specific B cells are sorted by fluorescence-activated cell sorting (FACS). The mRNA of those single cells is reverse-transcribed into cDNA followed by amplification of Ig heavy and light chains via PCR. The extracted antibody sequences can be cloned into a vector and ultimately introduced into an expression system. Finally, the resulting monoclonal antibodies are validated for their antigen specificity [5].
Abbreviations: Ag, antigen; EBV, Epstein–Barr virus; Ig, immunoglobulin; mAb, monoclonal antibody; V_H and V_L genes, variable heavy and light regions, respectively, of an Ig molecule.

by linking Fab fragments of two different specificities [57,58]. Furthermore, antibodies can be used as a shuttle to target antimicrobial drugs directly to the *S. aureus* cells [59–61]. This tool box for antibody engineering promises to be very helpful in designing protective anti-*S. aureus* monoclonal antibodies in the future.

Preclinical Studies on Antibodies Targeting *S. aureus* Biofilms

Despite the huge clinical impact of biofilm infections, research on antibodies targeting *S. aureus* infections often ignores biofilms during antigen selection and preclinical antibody testing. Nevertheless, there are several interesting *S. aureus* vaccine candidates with promising results in preclinical studies that we would like to highlight in this section, including adhesins, cell wall-modifying enzymes, surface glycopolymers, biofilm matrix components, and toxins (Figure 1, insert).

Adhesins

Staphylococcal adhesins, including MSCRAMMs, are among the most studied targets for antibody-based therapies [22]. Antibodies against adhesins exert their action via two mechanisms (Figure 2): (i) preventing the initial microbial adherence to abiotic as well as biotic surfaces [62–64], and (ii) coating the bacterial surface, thereby facilitating the clearance of an organism through bacterial **opsonophagocytic killing** [65,66]. In the context of biofilm vaccine development, interesting candidates are clumping factors A and B (ClfA and ClfB), and the fibronectin-binding proteins (FnBPA and FnBPB), as these cell wall-associated proteins are involved in biofilm formation and are widely distributed among the *S. aureus* clinical isolates, while the collagen-binding protein (Cna), biofilm-associated protein (Bap), as well as the *S. aureus* surface proteins C (SasC) and G (SasG) are present only in a subset of isolates [67].

The MSCRAMM ClfA promotes bacterial binding to fibrinogen. ClfA plays an important role in the colonization of implanted biomaterials or damaged endothelial surfaces at the site of endovascular infections [22,68]. Over the past 15 years, several monoclonal antibodies against ClfA were shown to block biofilm formation *in vitro* (Table 1) [62,66,69,70]. In animal infection models, anti-ClfA monoclonal antibodies protected from biofilm-associated infections (e.g., infective endocarditis, IE) as well as non-biofilm-associated infections (e.g., sepsis) [69,70]. In contrast, an anti-ClfA monoclonal antibody alone had only a moderate effect in a murine hematogenous implant infection, but in combination with antibodies against α-toxin (Hla), it effectively inhibited biofilm formation both *in vitro* and *in vivo* [62]. Moreover, a humanized anti-ClfA monoclonal antibody (Tefibazumab) conferred full protection against IE in rabbits when applied prophylactically [69].

FnBPs recognize fibronectin, fibrinogen, and elastin, and they promote intercellular accumulation and biofilm development (Table 1) [22]. Antibodies against FnBP inhibited *S. aureus* biofilm formation *in vitro*, and partially protected mice against endocarditis following sepsis [63,64].

Cell wall-modifying Enzymes

The surface-associated murein hydrolase autolysin, Atl, is a bifunctional enzyme that undergoes proteolytic cleavage to yield two cell wall-active enzymes, an amidase (Amd) and a glucosaminidase (Gmd). Both enyzmes are involved in bacterial cell separation after cell division, host extracellular matrix adhesion, and biofilm formation (Table 1) [71]. Polyclonal antibodies to Amd and Gmd inhibit biofilm formation and enhance opsonophagocytosis

Table 1. Molecular Targets for Antibody-based Therapies against *S. aureus* Biofilms in Preclinical Studies

Target[a]	*In vitro* anti-biofilm activities[b]	Protection in *ex vivo/in vivo* biofilm models[c]	Antibody[d]	Refs
Adhesins, including MSCRAMMs (microbial surface components recognizing adhesive matrix molecules)				
ClfA	Block FBG binding/agglutination of human plasma; displace FBG-bound bacteria; promote OPK	P: full – rabbit IE model T: partial (+ vancomycin) – rabbit IE model	Mu/mAb (mAb 12-9, 11H10), Huz/mAb (Tefibazumab)	[69,70]
FnBPA	Block FBN binding; promote OPK and nGr activation; reduce biofilm formation	Not tested	Mu/mAb	[63,64]
Cna	Block CN binding; displace CN from bacterial surface; promote OPK; block laminin and C1q binding	P: strong – sepsis model (fewer animals developed arthritis)	Mu/pAb, Mu/mAb	[133–135]
SasG	Reduce biofilm formation	Not tested	Ra/pAb	[136]
Cell wall-modifying enzymes				
Atl[1]	Inhibit biofilm formation; promote OPK	Not tested	Mu/pAb	[71,72,137][vii]
Atl-Amd	Promote OPK	Not tested	Mu/pAb	[72]
Atl-Gmd	Promote OPK; block cell division (binary fission); induce agglutination	P: strong – murine model of implant-associated osteomyelitis	IgG1 Mu/mAb (1C11)	[73]
IsaA	Promote nGr activation (oxidative burst) and OPK by nGr (UK-66); promote OPK in whole blood (hUK-66); promote nGr activation, but not phagocytosis (1D9)	P: partial, strain-dependent (1D9) – murine bacteremia model T: no (1D9) – murine bacteremia model; partial (UK-66) – catheter-related infection model	Mu/mAb (UK-66), Huz/mAb (hUK-66) Hu/mAb (1D9)	[138–140]
Glycopolymers				
WTA	Promote C3 deposition and OPK by nGr (Hu/pAb)	P: no – murine bacteremia model (Hu/mAb)	Hu/mAb, IgG Hu/mAb (THIOMAB)	[61,75]
CP	Promote OPK (Mu/mAb)	P: partial – rat endocarditis model (Ra/pAb); no protection – rat endocarditis model (Mu/pAb)	Mu/mAb, Ra/pAb, Mu/pAb	[77–79]
LTA	Promote OPK	Not published	Murine/human chimeric mAb (Pagibaximab®)	[141]
Matrix components				
PNAG/dPNAG	Promote OPK	P: partial – murine bacteremia model	IgG1 Hu/mAb (F598)	[29,65]
DNABII	Disrupt established biofilms	T: partial (+ daptomycin) – murine tissue cage infection model T: partial (+ vancomycin) – rat IE model	Native Hu/mAb (TRL1068)	[84,85]
Immune evasion proteins				
Spa	Neutralize Fcγ and V_H3+ Fab binding activities of Spa; Promote OPK in mouse and human blood	P: strong – murine sepsis model with renal abscess formation	Mu/mAb against Spa toxoid (3F6)	[142]
Toxins				
Hla	Neutralize toxin activity; modestly inhibit biofilm formation	P: complete – *ex vivo* porcine vaginal mucosa explants P: partial – murine hematogenous orthopedic implant infection (increased protection in combination with anti-ClfA 11H10 mAb)	Hu/mAb (MEDI4893)	[62,91,92]
LukAB	Neutralize LukAB-mediated cytotoxicity; inhibit LukAB binding to I domain of CD11b	P: partial – murine sepsis model (1:1 mixture of SA-15 and SA-17)	Hu/mAb (SA-13, -15 and -17)	[96]

Table 1. (continued)

Target[a]	*In vitro* anti-biofilm activities[b]	Protection in *ex vivo/in vivo* biofilm models[c]	Antibody[d]	Refs
Other proteins				
PhnD	Inhibit biofilm formation under shear flow (*S. aureus* and *S. epidermidis*), promote OPK by nGr	Not tested	Ra/pAb	[143]

[a]Protein names: ClfA, clumping factor A; FnBP, fibronectin-binding protein; Cna, collagen-binding protein; SasG, *S. aureus* surface protein G; Atl, autolysin: bifunctional enzyme that undergoes proteolytic cleavage to yield two catalytically-active proteins, an amidase (Amd) and a glucosaminidase (Gmd), which are both involved in peptidoglycan cleavage; IsaA, immunodominant staphylococcal antigen A; WTA, wall teichoic acid; CP, capsular polysaccharides; LTA, lipoteichoic acid; PNAG, poly-*N*-acetyl-β-(1,6)-glucosamine; dPNAG, deacetylated PNAG; DNABII, DNABII family proteins; Spa, staphylococcal protein A; Hla, α-toxin; LukAB, leukotoxins A and B; PhnD, subunit of alkylphosphonate ABC transporter.
[b]FBG, fibrinogen; OPK, opsonophagocytic killing; FBN, fibronectin; nGr, neutrophil granulocytes; CN, collagen.
[c]P, prophylaxis; T, therapy; IE, infective endocarditis.
[d]Abbreviations: mAb, monoclonal antibody; Hu/mAb, human mAb; Huz/mAb, humanized mAb; Mu/mAb, murine mAb; Ra/mAb, rabbit mAb; pAb, polyclonal antibodies; Hu/pAb, human pAb; Mu/pAb, murine pAb; Ra/pAb, rabbit pAb; IHF, integration host factor.

[71,72]. In addition, a monoclonal antibody against Gmd (1C11) reduced infection severity in a murine model of implant-associated osteomyelitis [73].

Glycopolymers

Staphylococcal cells are decorated with glycopolymers, including wall teichoic acids (WTA), peptidoglycan, lipoteichoic acids (LTA), and capsular polysaccharides (CP). These surface glycopolymers are recognized by serum antibodies and a variety of pattern-recognition molecules, including mannose-binding lectin. Anti-WTA antibodies facilitate complement C3 deposition via the classical pathway as well as opsonophagocytosis of laboratory and clinical *S. aureus* isolates by neutrophils (Table 1) [74,75]. Although a human monoclonal anti-WTA antibody was ineffective in preventing *S. aureus* infection in an intravenous mouse infection model, it showed promising *in vivo* results when conjugated to an antibiotic [61]. Further human monoclonal antibodies targeting WTA are currently being characterized [76]. However, to the best of our knowledge, anti-WTA antibodies have never been tested in biofilm-related infection models. Antibodies against the capsular polysaccharides promote opsonophagocytosis but yielded contradictory results when tested in a rat endocarditis model. While rabbit polyclonal antibodies conferred partial protection, murine antibodies were not protective [77–79].

Biofilm Matrix

The biofilm matrix has been recently brought into the focus of anti-biofilm vaccine research. This is in part due to the widely conserved nature of some of its components, making those components suitable vaccine candidates for protection against various human pathogens.

PNAG has been extensively evaluated as a potential vaccine candidate in relation to biofilm-associated infections (Table 1). In contrast to many *S. aureus*-specific biofilm factors, it is expressed among a variety of bacteria, fungi, and protozoa [80,81]. For instance, the immunological cross-reactivity of an opsonic antibody against *S. aureus* PNAG and *Escherichia coli* polyglucosamine has led scientists to investigate the possibility of developing a vaccine against both pathogens [80]. Several studies have highlighted the superiority of deacetylated PNAG (dPNAG) to PNAG in terms of immunogenicity and protection in animal models [80,82]. Anti-dPNAG immune sera provided efficient protection in a murine intraperitoneal [80,82], as well as

a bacteremia model [82]. More interestingly, the human IgG1 monoclonal antibody F598 (which binds both PNAG and dPNAG) has opsonic and protective activities against multiple microbial pathogens *in vivo* [65,81] and is currently undergoing preclinical and clinical assessments as a broad-spectrum antimicrobial therapeutic [83].

Bacterial DNA-binding proteins (DNABII family) have conserved homologs in a wide variety of bacterial species and are involved in a number of biofilm-associated infections [84,85]. They serve as adapter proteins for eDNA strands and hence stabilize the biofilm matrix (Figure 1, *insert*; Table 1) [86]. Loss of these scaffolding proteins, for instance by neutralization with specific antibodies, causes dispersal of the biofilm. The released bacteria regain their susceptibility to killing by antibiotics and are more easily cleared by phagocytes [86,87].

Recently, Estellés *et al*. generated a native human monoclonal antibody (TRL1068) recognizing a DNABII epitope conserved across a range of Gram-positive and Gram-negative bacterial species [84]. TRL1068 showed anti-biofilm efficacy in an *in vitro* biofilm assay, in a murine infectious implant model, as well as in a catheter-related biofilm infection model in rats [84,85]. However, as this antibody promotes biofilm dispersal, it is essential to eliminate the released bacteria to prevent subsequent dissemination to distant organs. Therefore, TRL1068 was proposed as a clinical candidate for the treatment of implant-associated infections in combination with standard-of-care antibiotics (Table 1) [84,85].

Toxins

Proteomic studies demonstrated that several pore-forming toxins [e.g., Hla, LukAB, and γ-hemolysin (HlgAB)], and immune evasion molecules (e.g., SCIN, and CHIPS) are produced within a biofilm *in vitro* and *in vivo*, some even in higher amounts than in planktonic cultures, whereas others, including the immune evasion protein A, are down-regulated [14,17,88]. The pore-forming toxins Hla, LukAB, and HlgAB lyse a range of host immune cells, including T cells, monocytes, and neutrophils [89], thereby torpedoing the anti-biofilm immune response. Neutralizing these toxins by monoclonal antibodies may enhance host defenses and facilitate clearance of planktonic and biofilm cells (Figure 1, *insert*).

Apart from destroying immune cells, Hla promotes biofilm formation *in vitro*, as well as *in vivo*, by disrupting the host epithelium, providing nutrients for bacterial survival through promoting host cell lysis, and facilitating bacterial cell-to-cell interactions [90,91]. The human monoclonal anti-Hla neutralizing antibody (MEDI4893) sterically inhibits binding of Hla to its cellular receptor ADAM10, effectively blocking pore formation [92]. It successfully abrogated *ex vivo* biofilm formation on porcine vaginal mucosa explants [91]. Considering that prophylactic treatment with MEDI4893 in a mouse model of *S. aureus* wound infection also promotes wound healing [93], this suggests that neutralization of Hla may be useful in biofilm-related *S. aureus* wound infections. MEDI4893 has been extensively tested in various biofilm and non-biofilm infection models (Table 1) [62,91,93].

The pore-forming toxin leukocidin A/B (LukAB) kills professional phagocytic cells, and together with Hla facilitates the persistence of staphylococcal biofilms [94]. Badarau *et al*. first reported the discovery of a highly potent neutralizing human IgG1 monoclonal antibody against LukAB (ASN-2), with a high-affinity antibody-binding site on the LukAB dimer [95]. In 2017, Thomsen *et al*. reported on three potently neutralizing, naturally

Table 2. Clinical Trials Involving Therapeutic Antibodies/Antisera against *S. aureus* Infections[a]

Target[b]	Name [Company; NCT number[c]]	Study design	Status (study result)	Intervention[d]	Refs
Single component					
ClfA (adhesin)	Tefibazumab (Aurexis®) [Inhibitex]	Randomized, double-blind, placebo-controlled trial of bacteremia patients receiving standard antibiotic treatment plus Tefibazumab (N = 63)	Phase II (failed)	Huz/mAb (IgG1)	[104]
	Tefibazumab (Aurexis®) [Inhibitex; NCT00198289]	Dose escalation study of Aurexis® in cystic fibrosis patients chronically colonized with *S. aureus* in their lung (N = 30)	Phase IIa (failed)	Huz/mAb (IgG1)	i
CP 5 and CP8 (capsular polysaccharides)	AltaStaph™ [Nabi Biopharmaceuticals; NCT00063089]	Randomized, double-blind, placebo-controlled trial involving adult *S. aureus* bacteremia patients receiving standard treatment plus Altastaph™ (N = 40)	Phase II (halted)	Polyclonal human IgG with high antibody titers against CP5 and CP8, purified from the plasma of healthy donors that have been vaccinated with StaphVAX®[e]	[105]
	AltaStaph™ [Nabi Biopharmaceuticals; NCT00066989]	Randomized, double-blind, placebo-controlled trial for prevention of nosocomial *S. aureus* infections in very low birth weight (VLBW) neonates (N = 206)	Phase II (failed)	Same as above	[106]
LTA (cell wall component)	Pagibaximab® [Biosynexus; NCT00631800]	Randomized, double-blind, placebo-controlled dose-ranging study on prevention of CoNS and *S. aureus* sepsis in VLBW neonates (N = 88)	Phase II (finished)	Murine/human chimeric mAb	[127]
	Pagibaximab® [Biosynexus; NCT00646399]	Randomized, double-blind, placebo-controlled study on prevention of staphylococcal sepsis in VLBW neonates (N = 1579)	Phase III (failed)	Murine/human chimeric mAb	[108]
WTA (wall teichoic acid)	DSTA4637S [Roche/Genentech; NCT03162250]	Randomized double-blind, placebo-controlled multiple-ascending dose study on safety, tolerability, and pharmacokinetics in *S. aureus* bacteremia (N = 24)	Phase Ib (ongoing)	THIOMAB™ antibody (Hu/mAb; IgG1)-antibiotic conjugate	[114,115]
PNAG (cell wall component)	SAR279356 [Sanofi-Aventis; NCT01389700]	Randomized, double-blind, placebo-controlled study to assess a single dose of SAR279356 in ICU patients on mechanical ventilation (N = 7)	Phase IIa (terminated due to difficulty in patient recruitment)	Hu/mAb	ii
Hla (toxin)	MEDI4893 (Suvratoxumab) [MedImmune LLC; NCT02296320]	Randomized, double-blind, placebo-controlled, single-dose, dose-ranging study in mechanically-ventilated adult subjects (N = 213)	Phase II (ongoing)	Hu/mAb (IgG1)	[111]
	AR-301 (Salvecin®) [Aridis Pharmaceuticals; NCT01589185]	Randomized, double-blind, placebo-controlled, single-dose study of AR-301 as an adjunctive therapy against severe *S. aureus*-related pneumonia (N = 48)	Phase IIa (successful)	Hu/mAb (IgG1)	iii
GrfA (ABC transporter)	Aurograb® [NeuTec Pharma Ltd/Novartis Pharma AG; NCT00217841]	Randomized, double-blind, placebo-controlled trial of patients with severe, deep-seated staphylococcal infections receiving vancomycin plus Aurograb® (N = 180)	Phase II (failed), development stopped	Single-chain antibody fragment (Fab)	[134,144]

Table 2. (continued)

Target[b]	Name [Company; NCT number[c]]	Study design	Status (study result)	Intervention[d]	Refs
Multicomponent					
ClfA (*S. aureus*), SdrG (*S. epidermidis*) (adhesins)	INH-A21 (Veronate®) [Inhibitex/Bristol Myers-Squibb; NCT00113191]	Randomized, double-blind, placebo-controlled study of INH-A21 for prevention of staphylococcal late-onset sepsis in VLBW infants (N = 1983)	Phase III (failed)	Pooled human Ig purified from the serum of donors with high titers against ClfA and SdrG	[113]
Hla, HlgAB, HlgCB, LukED, LukSF, LukAB (toxins)	ASN100 [Arsanis Biosciences GmbH; NCT02940626]	Randomized, double-blind, single-dose, placebo-controlled study of ASN100 for the prevention of *S. aureus* pneumonia in heavily colonized, mechanically-ventilated subjects (N = 354)	Phase II (halted)	Hu/mAb combination of ASN-1 (IgG1, cross-reactive mAb with affinity for Hla, HlgAB, HlgCB, LukED and LukSF) and ASN-2 (IgG1, mAb against LukAB)	[95,112]

[a]Data based on publications, review of sponsor website information, as well as clinical trial data accessible via www.clinicaltrials.gov on August 20, 2018.
[b]Abbreviations: ClfA, clumping factor A; LTA, lipoteichoic acid; PNAG, poly-*N*-acetyl-ß-(1,6)-glucosamine; Hla, α-toxin; SdrG, serine-aspartate repeat-containing protein G; HlgAB and HlgCB, γ-hemolysin AB and CB; LukED and LukSF, leukotoxin ED and SF; NA, data not available; VLBW, very low birth weight.
[c]ClinicalTrials.gov Identifier[iv].
[d]Antibodies applied in clinical studies were either murine, chimeric, humanized, or human (see Glossary). Abbreviations: mAb, monoclonal antibody; Hu/mAb, human mAb; Huz/mAb, humanized mAb.
[e]StaphVAX® is a bivalent *S. aureus* vaccine which contains the purified capsular polysaccharides (CP) types 5 and 8[v]. Its development was halted by Nabi Biopharmaceuticals due to failure in preventing *S. aureus* infections in kidney disease patients in a confirmatory phase III clinical trial[vi]. The company also halted the development of Altastaph™, as it is based on the same capsular polysaccharide technology as StaphVAX®.

occurring LukAB-specific human monoclonal antibodies, which reduced the bacterial load in a murine sepsis model (Table 1) [96].

Quorum sensing

Targeting quorum sensing, that is, bacterial cell density-dependent gene regulation, is a frequently promoted antivirulence strategy [97]. In *S. aureus* and other staphylococci, the quorum sensing system Agr controls virtually all known virulence factors, such as toxins, and secreted degradative enzymes [98]. In an exceptionally strict fashion, Agr controls the PSMs [99], which – as previously mentioned – trigger biofilm structuring and detachment [100]. Owing to this control, interfering with Agr quorum sensing results in the formation of thick undifferentiated biofilms [101]. Another less well-characterized potential quorum sensing system, LuxS, controls exopolysaccharide synthesis in a negative fashion [102,103]. Thus, interfering with quorum sensing in staphylococci by the use of monoclonal antibodies or any other means does not represent a promising/efficient anti-biofilm strategy.

In summary, several *S. aureus* vaccine candidates, including adhesins, cell wall-modifying enzymes, biofilm matrix components, and toxins, showed promising results in preclinical studies. To combat biofilm-related infections, future vaccination studies should aim at identifying and testing bacterial target structures expressed by both planktonic and biofilm cells, for instance by using proteomic approaches.

Clinical Trials on Antibodies Targeting *S. aureus*

Several of the targets described above, including ClfA, CP5 and 8, PNAG, Hla and HlgAB, have been tested as passive vaccines in clinical phase II and/or III trials (Table 2). However, none of them improved the clinical outcome in the treated patient cohorts. For instance, the anti-ClfA monoclonal antibody (Tefibazumab) failed to achieve statistically significant improvement of

clinical outcome in bacteremia and cystic fibrosis patients [104]. Similarly, polyclonal antiserum against CP5 and CP8 (AltaStaph™), as well as a monoclonal antibody against LTA (Pagibaximab®), failed in phase II and III trials, respectively (Table 2 and Box 1) [105–108]. Moreover, a phase IIa study on using an anti-PNAG monoclonal antibody in ventilated intensive care unit (ICU) patients was terminated.

This failure of trials using surface-directed monoclonal antibodies against adhesins and surface glyocopolymers forced *S. aureus* researchers to revisit *S. aureus* pathogenesis and potential correlates of protection. One lesson learned is that targeting a single adhesin is prone to failure, due to the high functional redundancy of these proteins. For instance, there are at least five fibrinogen-binding proteins in *S. aureus* [22]. Moreover, it has been suggested that adverse effects could be caused by antibody-induced agglutination, since large aggregates of bacteria in the blood may not be cleared by the host and could become trapped in various tissues, particularly in the lungs [109,110]. Finally, in contrast to other pathogens, opsonophagocytosis may not be the most important mechanism of protection, since this species produces a whole arsenal of toxins and immune evasion proteins that are decisive for pathogenesis. In consequence, current research and clinical trials are focusing on pore-forming toxins as targets for an antibody-based therapy (Table 2) [95,111,112]. MedImmune as well as Aridis Pharmaceuticals are testing human anti-Hla antibodies for prevention of *S. aureus* pneumonia [111].

Apart from a shift towards toxins, there is now a trend towards multivalent vaccines in order to combat the multifactorial nature of *S. aureus* pathogenesis [112,113]. For instance, Arsanis Biosciences has tested a combination of two human monoclonal antibodies (ASN-1 targeting Hla and four other bi-component leukocidins; ASN-2 targeting LukAB) in the ASN100 phase II clinical trial for the prevention of *S. aureus* pneumonia (Table 2) [95,112]. The trial was, however, recently halted due to insufficient efficacy. Another approach involves the use of monoclonal antibodies as a means of targeted delivery of antimicrobials. For instance, an antibody–antibiotic conjugate (AAC) specifically binding wall teichoic acid is currently used in a phase Ib clinical trial targeting *S. aureus* bacteremia patients [114,115] (Table 2).

Concluding Remarks and Future Perspectives

Although biofilm infections have been recognized as an important mediator of chronic infection associated with high morbidity and mortality, vaccine research has seemingly overlooked biofilms with regard to discovery and efficacy studies. A better understanding of the immune response against biofilms, and of how biofilms manipulate this response, is therefore essential for the development of protective staphylococcal vaccines (see Outstanding Questions). Nevertheless, in the reasonably near future, the identification and testing of new combinations of monoclonal antibodies, which are effective against planktonic as well as biofilm cells in a broad range of disease settings, will hopefully achieve more success than past attempts.

Ideally, the following factors should be considered while selecting potential biofilm-related antigens: (i) prevalence in clinical isolates [67], (ii) antigenic variability of the target protein [66,116,117], (iii) expression profiles of proteins within the biofilm *in vivo* [14,16], (iv) their pertinence to many different staphylococcal diseases, (v) immunological relevance, that is, accessibility to antibodies within the biofilm matrix, and (vi) ability to induce not only a strong but the correct (i.e., protective) type of immune response (governed by the right choice of adjuvant/route of antigen application) [118].

Outstanding Questions

What is the proteome (including surface proteins and secreted factors) of staphylococcal biofilms in *ex vivo* or *in vivo*-like conditions?

How does the adaptive immune system (antibodies, T cells) respond to biofilm as compared to non-biofilm infections?

How do biofilm-embedded bacteria modulate and subvert innate and adaptive defense mechanisms?

What are correlates of protection in biofilm infections – type1/3, type 2 or regulatory responses?

Are specific epitopes of antigens more effective in destabilizing biofilms and/or preventing biofilm formation?

Can the efficacy of monoclonal antibodies be enhanced by using a multivalent vaccine, or by combining antibodies with antibiotics or specific enzymes (such as nucleases, proteases)?

How well can 'reverse vaccinology', a genome-based unbiased discovery process for the prediction of candidate vaccine antigens, supplement traditional vaccine approaches?

In order to meet all or most of these criteria, multivalent vaccines seem to be the only strategy of choice for active as well as passive vaccination [18,44,66]. The most effective therapeutic approach for the biofilm lifestyle will likely require a combinatorial approach of bactericidal and immunostimulatory treatments. It may be an unrealistic goal to achieve a complete clearance of *S. aureus* from our body, bearing in mind that the microorganism is part of the human normal microbiota, and an expert in evading host immune defense, but rather aim at clinical protection, to reduce the severity of staphylococcal infections, and prevent chronification.

Recapitulating the unsuccessful clinical trials for a passive *S. aureus* vaccine, several hurdles can be named: (i) the multiplicity and redundancy of *S. aureus* virulence factors, which challenges the selection of protective antigens, (ii) the production of numerous immune evasion factors, including protein A, (iii) insufficient knowledge of the nature of protective immunity against *S. aureus* infection, and (iv) a lack of successful transition from animal models to human clinical trials [109,119,120]. One possible explanation for this failed transition could be the use of naïve animals, whereas humans are immunologically primed against *S. aureus*. This may explain why huge effects of different passive or active vaccination strategies in animals cannot be reproduced in humans. If these points are considered in antigen selection processes and subsequent preclinical tests, we will hopefully be more successful in the near future in developing a protective passive vaccine against this notorious pathogen.

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Resources

[i]https://clinicaltrials.gov/ct2/show/NCT00198289

[ii]https://www.sanofi.com/en/science-and-innovation/clinical-trials-and-results/our-disclosure-commitments/pharma/-/media/Project/One-Sanofi-Web/Websites/Global/Sanofi-COM/Home/common/docs/clinical-study-results/PKD11791_summary.pdf

[iii]https://aridispharma.com/ar-301/

[iv]https://clinicaltrials.gov/

[v]https://clinicaltrials.gov/ct2/show/NCT00211913?term=StaphVAX&rank=1

[vi]www.sec.gov/Archives/edgar/data/72444/000119312505214434/dex99.htm

[vii]https://www.ncbi.nlm.nih.gov/pubmed/27044299

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