Mechanisms to suppress or enhance phagocytosis of staphylococci

Annemarie Kuipers

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Mechanisms to suppress or enhance phagocytosis of staphylococci

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|Chapter 1

General introduction | *S. aureus* under stress by complement and coagulation

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Bacteria under stress by complement and coagulation

*Equal contribution to review

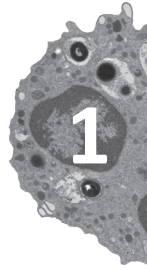
Staphylococcus aureus

Staphylococcus aureus is a Gram-positive bacterium, which lives in 30% of the human population as a commensal on the skin and mucosal membranes[1]. However, *S. aureus* is able to cause community- and hospital-acquired diseases, ranging from mild skin infections to bacteremia, sepsis and endocarditis. The typically infected persons used to be elderly and immunocompromised patients but, due to emergence of the very virulent community-associated methicillin-resistant *S. aureus* (MRSA) strains, also young immunocompetent persons are affected nowadays. MRSA is a potent pathogen causing up to 46% of deaths among hospital-acquired bacteremia in the Netherlands, showing the highly virulent potential of the pathogen[2]. *S. aureus* strains produce a viscous extracellular glycoconjugate (biofilm) that allows them to adhere to smooth surfaces like catheters[3]. Infections by *S. aureus* can occur in almost every organ in the body and are often associated with skin, soft tissue, respiratory tract, bone, joint, urinary tract and endovascular disorders. More serious and life-threatening *S. aureus* infections are bacteremia, endocarditis, sepsis, metastatic infections and the toxic shock syndrome. The majority of these life-threatening infections, however, occurs in persons with a compromised defense mechanism[1].

The cell envelope of *S. aureus* consists of a thick peptidoglycan (PG) layer, which is a common cell wall component of almost all bacteria and important for cell integrity. It is composed of polysaccharide strands of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acids (MurNAc) that are linked by short peptides (reviewed in [4]). Gram-positive bacteria possess one phospholipid membrane covered by a thick PG layer (20-80 nm) (reviewed in [5]) that contains complex polysaccharides and teichoic acids linked to the peptidoglycan (wall teichoic acids, WTA) or the membrane (lipoteichoic acids, LTA)[6]. Next to these conserved cell wall structures, *S. aureus* evolved a plethora of extracellular and cell-associated virulence factors[7,8]. Together these virulence proteins are critical for bacterial adherence, cell internalization, host tissue invasion and evasion from the immune system**.**

The innate immune response against *S. aureus*

The human innate immune system is our first line of host defense against invading bacteria. The main function of the innate immune system is to swiftly recognize and eliminate bacteria without damaging host tissues[9]. The system consists of several cellular and humoral plasma components that contain specialized mechanisms to both recognize and eliminate the invading pathogen. Rapid elimination of pathogenic *S. aureus* critically depends on activation of both the complement system and phagocytic cells of the innate immune system[9,10]. During local infection, circulating phagocytes (neutrophils) are attracted from the blood to the site of infection by chemotactic components from the complement system but also through chemotactic signals from other phagocytes[10]. The chemotactic components are bound by specialized receptors on the cells and cause them to migrate upstream the gradient of components. Phagocytosis is also essential for the direct eradication of systemic *S. aureus* infections by phagocytes that reside in filtering organs like the liver (kupffer cells, macrophages)[11]. Both circulating and resident phagocytes have specialized killing mechanisms to clear *S. aureus* intracellular.



Complement activation on bacteria

Complement is an integral part of the innate immune system, our primary host defense barrier against invading bacteria[12,13]. Whereas the complement reaction exclusively takes place on the bacterial surface, the resulting cleavage products are either linked to the surface or released in fluid-phase. The released chemotactic peptides attract phagocytes from the blood to the site of infection[14], while the massively deposited C3 fragments enhance bacterial recognition by phagocytes and trigger phagocytosis (**figure 1**). Finally, the pore-forming complex (Membrane Attack Complex or MAC) that inserts into the membrane[15] results in direct bacterial killing. Complement activation is a step-wise process in which protein binding and cleavage events occur in a well-defined order[13,16]: first, the recognition molecules of the three complement pathways (classical, lectin, alternative) bind to the bacterial cell surface (**figure 2**); second, they activate associated serine proteases that form C3 convertase enzymes (**figure 3A**); third, C3 convertases cleave the central complement protein C3 to label the target surface with C3b and amplify the labeling process; fourth, C5 convertases convert C5 into C5a and C5b; fifth, C5b generates the MAC (C5b-9) (**Figure 3A**). The recognition phase is important for the specific activation of complement on target cells. Recognition molecules generally bind evolutionarily conserved structures that are only present on microbes and absent on host cells.

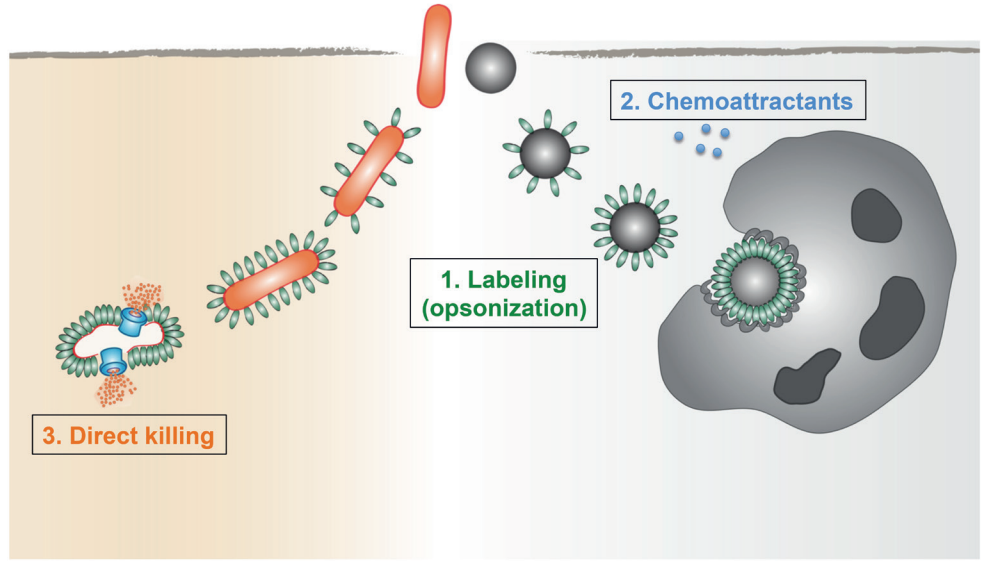


Figure 1. Anti-bacterial effector functions of the complement system. Complement activation on invading bacteria results in **1**) labeling of bacteria with C3-derived products to mediate phagocytosis by immune cells; **2**) release of potent chemoattractants (C5a) that attract phagocytes to the site of infection; **3**) direct killing of Gram-negative bacteria via the Membrane Attack Complex. Gram-negative bacteria indicated in orange, Gram-positive bacteria as grey circles.

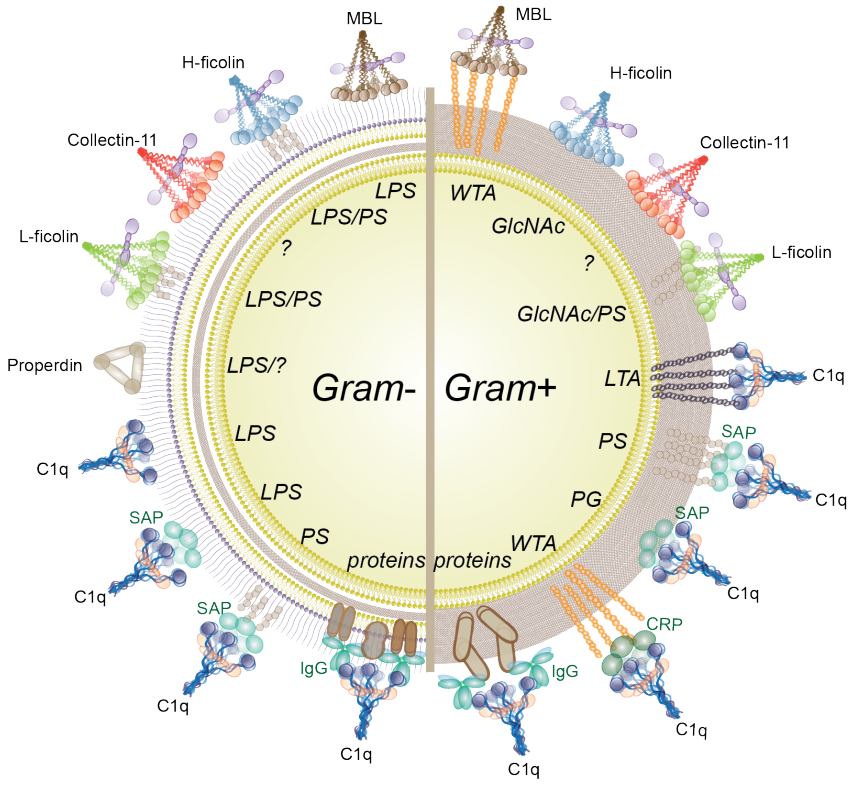


Figure 2. How bacteria are recognized by complement. Complement recognition molecules of the CP (C1q), LP (MBL, ficolins, collectin-11) and AP (properdin) bind to various structures on Gram-positive and -negative bacteria (recognition motifs in italics). Next to direct binding, C1q also indirectly recognizes bacteria via antibodies, CRP or SAP. MBL: Mannose-Binding Lectin, SAP: Serum Amyloid P, CRP: C-Reactive Protein, IgG: Immunoglobulin G. LPS: Lipopolysaccharide, PS: polysaccharides, WTA: Wall Teichoic acid, GlcNAc: N-acetyl glucosamine, LTA: Lipoteichoic acid.

Complement recognition of bacteria. It has long been recognized that isolated PG, which is unique to bacteria, is a potent activator of the complement system[17,18]. In the context of living bacteria, however, PG is less exposed; it is hidden underneath the outer membrane (OM) of Gram-negative bacteria or shielded by PG-anchored polysaccharides and teichoic acids in Gram-positives. Therefore, complement also recognizes other conserved bacterial structures (**figure 2**). The recognition molecules of the lectin pathway (LP) are the mannose binding lectin (MBL), ficolins (Ficolin-1, Ficolin-2, and Ficolin-3) and collectin-11[19–22]. These large multimeric complexes resemble a 'bunch of tulips' since they hold a collagen-like domain build up from three identical polypeptide chains that assemble in a coiled-coil structure at the base and multiple specific carbohydrate binding domains at the top. MBL and collectin-11 contain a C-type carbohydrate-recognition domain (CRD)[23] that allows binding to carbohydrate and glycoconjugate structures on many different microbes (reviewed in [24]). The main bacterial targets of MBL are LPS in Gram-negative and WTA in Gram-positive bacteria[25–27]. Collectin-11 was recently identified and described to bind *Escherichia coli*, *Pseudomonas aeruginosa*[28] and *Streptococcus pneumoniae*[29]. In ficolins, the CRD consists of

a fibrinogen-like domain that recognizes both sugar motifs[30] and acetylated groups present in for instance GlcNAc and GalNAc[19]. The binding to GlcNAc, a major constituent of PG, is believed to be important for the binding to Gram-positive bacteria[18].

The C1q molecule, which is structurally similar to MBL and ficolins in the LP, mediates activation of the classical pathway (CP). C1q is classically known to bind to Fc regions of IgG or IgM antibodies on the microbial surface. For some bacteria like *Neisseria meningitidis*, antibodies are essential for an effective complement response[31]. Furthermore, C1q binds bacteria via pentraxins, evolutionarily conserved plasma proteins that recognize foreign antigens and altered-self ligands. The major pentraxins in human plasma are the short pentraxins C-reactive protein (CRP) and serum amyloid P (SAP) (reviewed in [32]) and the long pentraxin 3 (PTX3)[33]. CRP interacts with phosphocholine (PC) that is present in a number of bacterial structures including the teichoic acid of *S. pneumoniae*[34]. SAP binds a variety of bacterial cell wall structures such as carbohydrates, LPS, and peptidoglycan[35,36]. Alternatively, C1q can bind directly to the bacterial surface to activate the CP. C1q was found to bind to a variety of microbial structures including the lipid A region of LPS[37] and different outer membrane proteins on Gram-negative bacteria[38,39], and LTA on Gram-positive bacteria[38]. Moreover, C1q can bind to various carbohydrates[40], which potentially mediates direct binding to bacteria.

Recently it was shown that the alternative pathway (AP), generally viewed as an amplification mechanism of the CP and LP, also directly recognizes bacteria via the properdin molecule. It has been reported that properdin, a stabilizing protein for the convertases of the AP, functions as a pattern recognition receptor (PRR) for bacteria[41]. Properdin binds to LPS of certain Gram-negative bacteria but also to LPS-defective *E. coli* and *Salmonella typhimurium*. Also, properdin was reported to bind and activate complement on *Chlamydia pneumoniae*[42]. However, since other studies have questioned these data, the direct recognition of bacteria via the AP is still under debate[43]. Of note, activation of the AP can also occur at a low-level via the spontaneous 'tick-over' process in which hydrolysis of C3 creates the C3b-like molecule $C3H_2O$[13]. This activation process is down regulated on host cells but quickly amplified on bacterial cells.

Thanks to the large variety of recognition mechanisms, almost all bacteria will be recognized by the complement system. Many studies have shown that the relative importance of each pathway may be different for every bacterium. Importantly, the contribution of each pathway may change during an active infection due to upregulation of the acute phase response (CRP and MBL) and the (increased) production of antibodies. Recently, an interesting study reported that binding of MBL to WTA of *Staphylococcus aureus* occurs exclusively in infants with low levels of anti-WTA antibodies. In adults, higher levels of anti-WTA antibodies compete with MBL for WTA binding and trigger activation of the classical pathway[27]. Whether such a pathway-shift results in a more effective complement response is presently unknown.

Complement activation on bacteria. Whereas the initial phase of complement recognition is strictly dependent on the composition of the bacterial surface, the next steps of the complement pathway occur similarly on all target surfaces (**figure 3A**). The recognition molecules do not possess enzyme activity but circulate in complex with serine proteases responsible for activation of the proteolytic cascade. The recognition molecules of the

LP (MBL, Ficolins and Collectin-11) circulate in complex with the MBL-associated serine proteases (MASPs) while C1q is complexed with the C1r and C1s proteases. The homologous MASP-2 and C1s proteases can both cleave C4 and C2 to generate a C3 convertase enzyme that consists of surface-bound C4b and protease C2a (**figure 3A**). This convertase catalyzes the covalent deposition of C3b onto the bacterial surface and release of the small anaphylatoxin C3a. The covalent binding of C3b, a critical step in the complement response, is mediated by its reactive thioester. Thanks to the crystal structures of C3 and C3b[44], we now understand how this thioester is hidden within C3 but becomes exposed in C3b as a result of extensive conformational changes (**figure 3A**). Although the acyl group of the thioester is thought to form a covalent bond with any hydroxyl or amine group[44,45], this reaction is probably less random than generally believed[45,46]. Most C3b is linked by an ester bond, which indicates that it reacts better with hydroxyl groups than amine groups[46]. Binding occurs preferentially to hydroxylated sugar groups, but also to amino acids in proteins of which tyrosine, threonine and serine show the highest reactivity (Tyr>Thr>Ser)[47].

The deposited C3b molecules initiate the alternative pathway (AP) amplification loop because C3b itself forms the basis of the AP C3 convertase enzyme (C3bBb). Surface-bound C3b forms a complex with Factor B, which is then converted by Factor D[16]. The resulting C3bBb enzyme converts new C3 molecules into C3b (**figures 3A, B**) and thus rapidly amplifies the number of deposited C3b molecules. This is crucial for initiation of the terminal pathway, since high densities of C3b are required to change the affinity of the convertase for C5 [48], which can then cleave C5 into the chemo-attractant C5a, and soluble C5b that initiates assembly of the MAC.

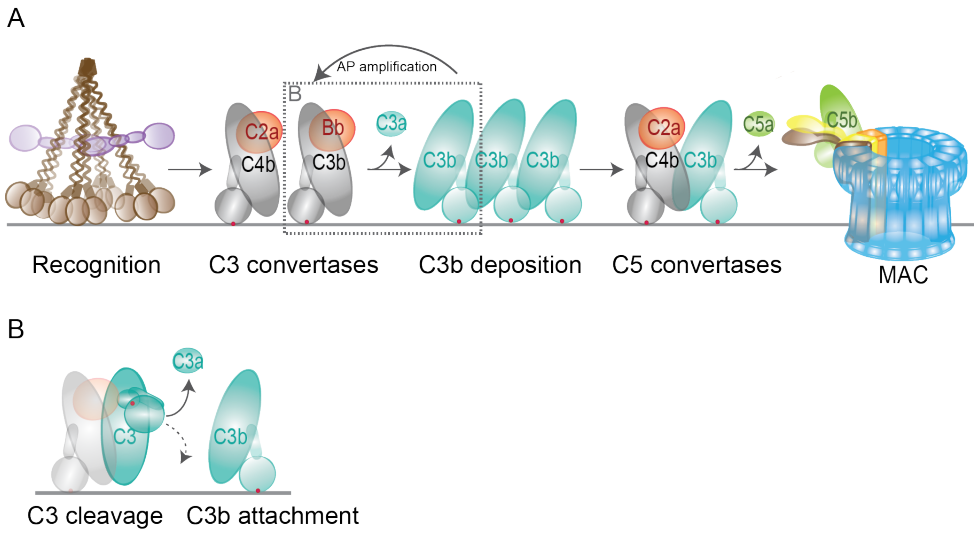


Figure 3. The complement reaction on bacteria. A. Recognition molecules initiate the complement cascade by their associated serine proteases (C1s and MASP-2, purple) that cleave C4 and C2 to generate a C4b2a complex on the bacterial surface. This complex is a C3 convertase that cleaves C3 into the released anaphylatoxin C3a and C3b, which binds covalently to the bacterial surface. C3b molecules initiate formation of another C3 convertase (C3bBb) that amplifies the C3b labeling process (AP amplification). C3b molecules also generate C5 convertases by binding onto or near the C3 convertases. This initiates a substrate switch, which causes convertases to cleave C5 into soluble C5a and C5b that forms a complex with C6-9 to generate the MAC. This figure was partly modified from[16]. **B**. Conversion of C3 by the C3 convertase. C3 holds a reactive thioester bond (red dot) that is hidden within the C3 molecule but exposed upon its cleavage into C3b. The acyl group of the thioester moiety forms a covalent bond with a hydroxyl or amine group, which allows covalent attachment of C3b to the target surface.

Complement effectors. Complement activation products can exert stress to bacterial cells, either indirectly by supporting cellular immune responses or directly by damaging bacterial membranes. Direct stress of complement to bacterial cells is mainly exerted through formation of the MAC that kills Gram-negative bacteria. The MAC is a multiprotein complex that is comprised of single copies of C5b, C6, C7, and C8 together with 12-18 copies of C9 [15]. A very important role of complement is to facilitate phagocytosis of bacteria by labeling (or opsonizing) their surface with C3b and its degradation product iC3b, which are recognized by complement receptors 1 (CR1), 3 (CR3), 4 (CR4), and CRIg on phagocytic cells[11,49]. Also, the release of anaphylatoxins C5a and C3a is important to trigger a pro-inflammatory status. Moreover, neutrophils and monocytes (C5a), but also eosinophils and mast cells are attracted to the site of infection through interaction with the C5a (C5aR) or C3a receptor (C3aR)[10]. Moreover, complement activation can orchestrate adaptive immunity by enhancing antigen presentation via interaction of the C3b degradation product C3d with CR2 on B cells and follicular dendritic cells[50]. In addition, complement activation products influence T cell immunity via stimulation of antigen presenting cells (APCs) and T cells[51].

Phagocytosis

Although phagocytic cells can recognize invaders directly through PRRs, phagocytic uptake is greatly enhanced through the interaction of antibodies or complement on bacterial surfaces with Fc and complement receptors (CRs) on the phagocyte cell surface[52]. Phagocytic cells carry complement receptors that bind C3b and C3b fragments on the pathogenic surface[13]. C3b is bound by complement receptor (CR) 1 (CD35) which, besides promoting phagocytosis, acts as a cofactor in the inactivation of C3b by factor I. CR3 (CD11b/CD18) and CR4 (CD11c/CD18) recognize and promote phagocytosis of iC3b-coated surfaces. Furthermore, iC3b and C3dg are bound by CR2 (CD21) mediating B-cell activation and hereby increasing the antibody response[50]. Recently, a novel complement receptor has been discovered on Kupffer cells (KCs)[11]. KCs are macrophages residing in the liver were they play a dominant role in clearing the bloodstream of pathogens and “non-self” particles. The receptor has been identified and characterized as the complement receptor of the immunoglobulin superfamily (CRIg). CRIg binds C3b and iC3b and hereby mediates clearance of C3-opsonized pathogens. Next to complement receptors, phagocytic cells also engulf bacteria via Fc receptors that bind to antibodies on the bacterial surface. The most abundant and important activating Fc receptor expressed on human neutrophils is Fc gamma receptor (FcγR)IIa, followed by FcγRIIIb. Both receptors are primarily capable of binding Immunoglobulin G (IgG), only when aggregated in complex with antigens[53]. The different subclasses of IgG (IgG1, IgG2, IgG3, IgG4, in order of abundance within human serum) vary in their ability to bind FcγRs[54]. IgG2 and IgG4 have been described to have reduced affinity for FcγRIIa compared to IgG1 and IgG3 and seem incapable of associating with FcγRIIIb[53,54].

Bacteria under stress by coagulation

The complement and coagulation systems are two ancient proteolytic cascades in plasma that play important roles in host defense and hemostasis respectively. Both systems are more than 400 million years old and probably evolved from a common ancestor, explaining the significant overlap in protein domains[55]. The primary function of the coagulation system is to respond to damage of blood vessels via formation of fibrin, which seals wounds and stops bleeding (hemostasis). Although clotting is a well-known immune defense mechanism in invertebrates, its role in mammalian host defense is largely unknown. However, multiple lines of evidence now suggest that coagulation is important for the early innate immune response since it supports the inflammatory response, generates antimicrobial peptides and induces local entrapment of bacteria in fibrin clots[56,57].

The coagulation cascade can be activated via different pathways that all converge in a common pathway for clot formation[58]. Two pathways can trigger coagulation: the contact system (intrinsic pathway) and the tissue factor (extrinsic) pathway. The tissue factor pathway responds to blood vessel damage via exposure of tissue factor, a subendothelial protein that is normally shielded from contact with blood components. Since this pathway plays no clear role in host defenses, it will not be further discussed here. Microbe-specific activation of coagulation was suggested to occur via the FXII-dependent contact–driven pathway, which results in formation of the pro-inflammatory peptide Bradykinin (BK) (**figure 4A**) and clotting via activation of factor XI (**figure 4B**).

Contact activation on bacteria

The contact system is activated upon binding of FXII to negatively charged structures, as was demonstrated in coagulation assays using dextran sulfate or silica[59]. Since these structures are abundantly present on bacteria, FXII can bind to bacterial surfaces[60]. Target binding of FXII results in low-level auto-activation into FXIIa, which then converts prekallikrein (PK) into active plasma kallikrein (**figure 4A**)[59]. Kallikrein can cleave the associated high molecular weight kininogen (HK) to release the pro-inflammatory peptide bradykinin (BK). Furthermore, kallikrein amplifies the contact system by cleavage of FXII[61]. At least 75 to 90% of PK circulates in an equimolar complex with HK[62], which is important for effective cleavage of PK by FXIIa. The activated form of HK (HKa) has an increased capacity to bind negatively charged surfaces and provides a surface for further activation of the cascade. *E. coli* curli and *S. typhimurium* fimbriae can activate the contact system due to interactions with FXII and HK. All contact factors were assembled on these fibrous structures and mutant strains lacking either curli or fimbriae did not activate the cascade[60]. Also LPS was described to provide a surface for contact activation by binding to domain 5 in HK, which is responsible for binding to negatively charged surfaces[63]. For *S. aureus* it was proposed that negatively charged teichoic acids induce contact activation[64]. Apart from surface-associated molecules, fluid-phase bacterial products can also initiate the contact system. For instance, LPS is released from the bacterial surface during cell growth and can then activate the contact system by binding HK in solution[63]. Also, it has been described that excreted bacterial polyphosphates can trigger coagulation through the contact pathway by binding to FXII and to a lesser extent to HK, depending on the length of the polymer chains[65].

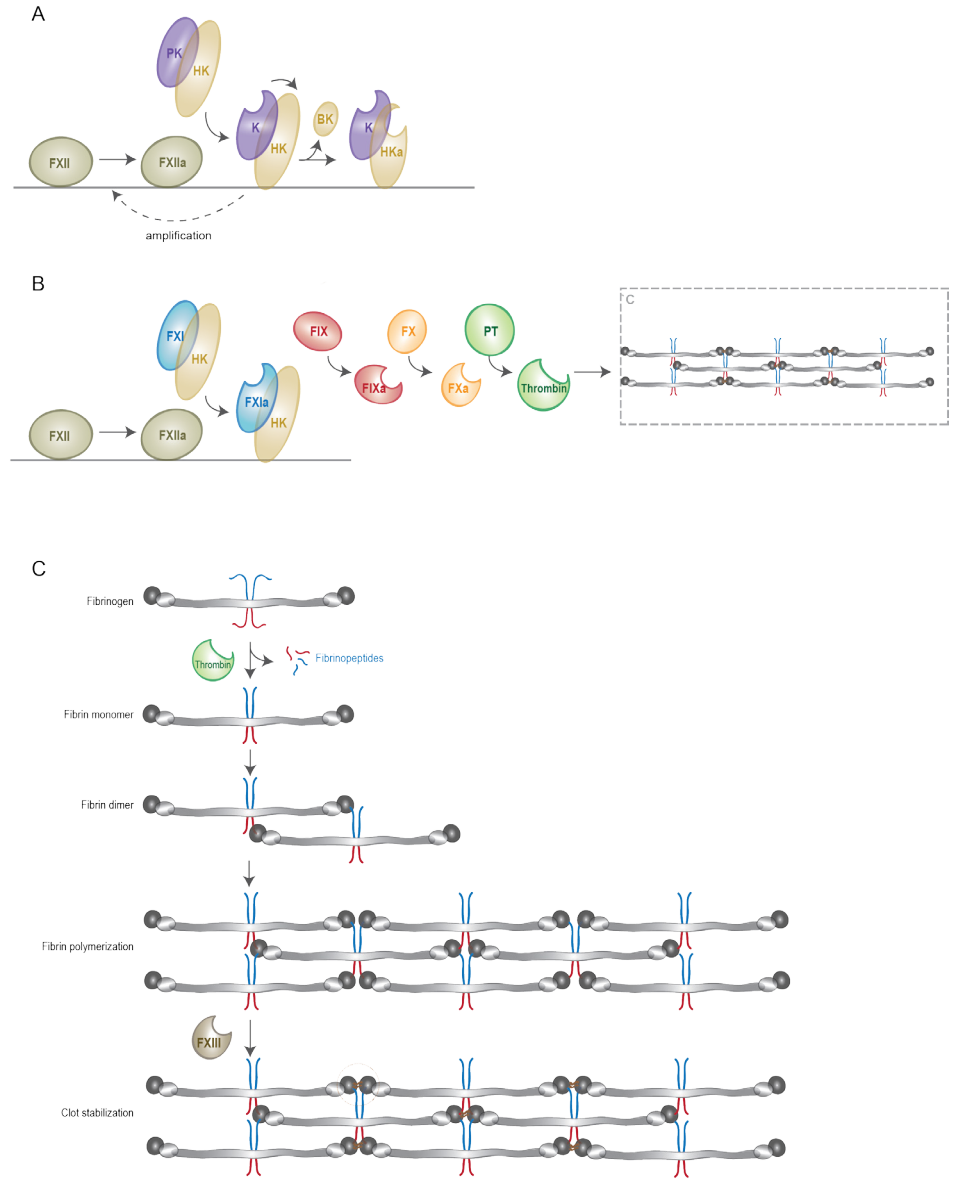


Figure 4. Activation of coagulation on bacteria. The contact activation pathway is initiated by the binding of FXII to negatively charged bacterial structures. FXII is then auto-activated into FXIIa, which either triggers the kallikrein-kinin system (**A**) or the clotting cascade (**B**). **A**. FXIIa converts PK, which circulates in complex with HK, into active kallikrein. Kallikrein cleaves HK to release BK and activates FXII to amplify the contact system. **B**. FXIIa cleaves FXI, which also circulates in complex with HK. FXIa subsequently converts FIX into FIXa. FIXa will then trigger the common pathway of coagulation by converting FX into FXa, which leads to conversion of prothrombin into thrombin that cleaves fibrinogen. **C**. Formation of fibrin clots. Activated thrombin cleaves fibrinogen into fibrin monomers that are capable of interacting with adjacent fibrin molecules and thereby form long fibers and finally a fibrin clot. This clot can be stabilized by FXIII or thrombin-activated FXIIIa.

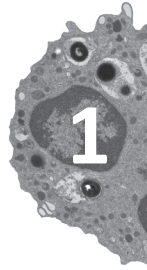
Next to its role in cleavage of PK and HK, contact activation also results in clotting (**figure 4B**). Surface-activated FXIIa can also cleave coagulation factor XI, a homologue of PK that circulates in complex with HK as well. As explained in **figure 4B**, activated FXI (FXIa) triggers the common pathway of coagulation in which subsequent activation of FIX, FX and prothrombin results in thrombin-catalyzed cleavage of fibrinogen (**figure 4B,C**). Fibrinogen is a highly abundant (4 mg/ml) and large plasma protein (340 kD) that consists of two sets of three polypeptide chains (Aα , Bβ and Cγ) organized in three distinct domains: two outer D-domains and a central E-domain connected by coiled coil segments[66]. Thrombin drives fibrin formation by cleavage of peptide bonds in both the N-terminal Aα chain and the N-terminal Bβ-chain that are located in the E-domain (**figure 4C**). Cleavage of the Aα-chain results in release of fibrinopeptide A and exposure of a polymerization site known as $E_{A.}$ This newly exposed E_A-site subsequently interacts with the constitutively exposed D_a-site on the β-chain segment of the D-domain of adjacent fibrin(ogen) molecules, leading to formation of a staggered overlapping double-stranded fibrin fibril. Cleavage of the Bβ-chain by thrombin and the associated release of fibrinopeptide B is much slower and leads to exposure of the E_B polymerization site that interacts with constitutive D_b-sites on the β-chain region of the D-domain. This interaction induces a conformational change in the D-domain that allows lateral association of fibrin fibers[67]. Fibrin fibers are subsequently stabilized by factor XIII or thrombin-activated FXIIIa[68], a transglutaminase that introduces covalent intermolecular bonds between C-terminal lysine and glutamine residues of adjacent γ-chains. Recently it was shown that contact activation by bacterial fimbriae and curli indeed results in cleavage of fibrinogen and subsequent fibrin formation[69].

The contact system: an innate recognition pathway?

Due to its redundant function for efficient hemostasis, the contact system has been proposed to be part of the innate immune system. Interestingly, the recognition mechanisms of the contact system seem less specific than those of the complement system and innate immunity in general. In order to respond to a large group of microbes, the innate immune system uses a variety of proteins that recognize evolutionarily conserved structures on non-self surfaces. For example, toll-like receptor 4 (TLR4) recognizes Gram-negative bacteria by binding to LPS, the formylated peptide receptor (FPR) responds to all living bacteria by sensing formylated peptides (exclusively produced by bacteria)[14] and the complement system uses a variety of lectins to bind conserved bacterial sugars or teichoic acids. In contrast, the contact system is not activated by conserved bacterial structures but recognizes bacteria via their negatively charged surface. Still there appears to be some specificity since contact activation does not normally occur in the circulation and seems to be limited to foreign surfaces or human cells in a procoagulant state.

Host defense functions of coagulation on bacteria

The coagulation system is recognized to be important to the immune response of invertebrates. Several invertebrate species contain a coagulation cascade that senses invading microorganisms and neutralizes them via clotting[70]. Horseshoe crab clotting factors are stored in the granules of hemocytes (phagocytic cells) that are released upon sensing



bacterial components like LPS. This triggers a proteolytic cascade that eventually leads to cleavage and polymerization of the clottable protein coagulogen that embeds bacteria in insoluble aggregates[70]. In addition to clottable proteins, the immune system of invertebrates uses transglutaminases that are highly homologous to FXIII to stabilize clots containing bacteria. In *Drosophila melanogaster*, a lack of transglutaminases led to an immune defect since microbes were no longer entrapped in clots[71].

In contrast to invertebrates, the mammalian coagulation system was long considered to be exclusively important for hemostasis. However, several studies now indicate that coagulation factors also contribute to the effective elimination of bacteria in mammals. Activation of coagulation on bacteria triggers multiple biological effects important for host defense. While some of these effectors induce direct stress on the bacteria, others support the cellular immune response. Before we zoom in to the direct antimicrobial effects of coagulation, we will briefly summarize the indirect immune functions of coagulation. One important consequence of coagulation at the site of infection is the recruitment of innate immune cells via BK that binds kinin receptors (B1R and B2R) on endothelial, smooth muscle and innate immune cells[56]. BK induces vascular leakage and interacts with macrophages to release chemo-attractants. Furthermore, a number of immune cells express protease-activated receptors (PARs) that are cleaved by coagulation proteases (thrombin and FXa) to initiate inflammation[72]. Finally, activation of fibrinogen is accompanied by the release of the fibrinopeptides A and B, which are also chemo-attractants[73].

Fibrin formation. The best-defined direct action of coagulation on bacteria is the formation of fibrin at the site of infection. When bacteria become immobilized inside the fibrin network, they are prevented from spreading into the surrounding tissues. It has been known for decades that this local entrapment reduces bacterial dissemination into the bloodstream. Mice deficient in fibrinogen are therefore more susceptible to infections with GAS due to decreased fibrin production[74]. For some bacteria like *S. aureus* and *E. coli*, it was found that this immobilization inside a fibrin clot is FXIII dependent[71] and that bacteria are directly attached to the fibrin fibers via multiple binding sites cross-linked by FXIII. In the absence of FXIII, they appear to be loosely assembled within the clot without direct interaction with the fibers. Furthermore, it has been described that FXIII triggers entrapment of GAS at the site of infection, which subsequently leads to killing of the bacteria due to generation of plasma-derived antimicrobial peptides[75]. In a murine skin infection model these bacteria were also shown to cluster within fibrin networks in the presence of FXIII, whereas in the absence of FXIII they are distributed throughout the infection site. This leads to a higher influx of neutrophils and thereby increased inflammation. Furthermore, a systemic response, and therefore bacterial dissemination, was induced when FXIII was absent[75]. Furthermore, fibrin polymerization mediates clearance of *E. coli* and *S. aureus* from the peritoneal cavity and limits the growth of *Listeria monocytogenes* in the liver[76].

Antimicrobial peptides. Another direct action of coagulation in host defense against bacteria is the production of antimicrobial peptides (AMPs). First of all, BK itself was found to exhibit antimicrobial activity *in vitro* although it has been debated if this is relevant *in vivo*[56]. Also, coagulation proteins are sensitive to cleavage by neutrophil-derived proteases resulting in peptides with antimicrobial activity[77]. For instance, thrombin-derived C-terminal peptides (TCP) were produced when fibrin clots or human plasma was incubated with neutrophil

elastase, which could induce lysis of microbial membranes[78]. TCPs were found to be antibacterial against the Gram-positive *S. aureus*, as well as to Gram-negative species *P. aeruginosa* and *E. coli*. Membrane permeabilization by the TCP GKY25 was confirmed by electron microscopy[78]. Although it was shown that GKY25 acts on the bacterial membrane, the precise mechanism remains to be elucidated. HKH20, a peptide derived from domain 5 of HK, also displays antibacterial activity against both Gram-positive (*S. aureus* and *Enterococcus faecalis*) and -negative species (*E. coli* and *P. aeruginosa*)[77,79]. Antibacterial activity of this peptide and its truncated variants was correlated to the ability to cause leakage in model lipid bilayers, indicating that disruption of membrane integrity is the mechanism that leads to HKH20-mediated bacterial killing[79]. The effect of HKH20 is similar to that of the HK domain 3 derived peptide, NAT26. This peptide kills bacteria that activate the contact system, GAS AP1, *S. aureus* Cowan I, and *S. typhimurium* SR11B[56] and their cell walls were disintegrated when analyzed by electron microscopy. Furthermore, also this peptide causes leakage of anionic liposomes by disrupting the membrane[56]. Finally, the fibrinogen-derived peptide GHR28 also exhibits antibacterial activity against the Gram-positive species *Streptococcus agalactiae* (GBS) and *S. aureus*, but not against *E. faecalis* and the Gram-negative bacterium *E. coli*[80].

Crosstalk between complement and coagulation

There is a significant amount of crosstalk between the complement and coagulation systems and this has been reviewed in a number of excellent papers[81,82]. Here we would like to highlight a few of these interactions that, in our opinion, are of special interest in light of host defenses against bacterial infections. First, it was reported that several complement proteases activate the final stage of coagulation to form fibrin clots. A major role has been suggested for the MASPs, which circulate in complex with MBL or ficolins and are thus activated on bacterial surfaces via pattern recognition. Next to the role of MASP-2 in activation of complement, this protease was also found to cleave prothrombin into thrombin thereby promoting fibrin clot formation[83]. Although the prothrombin turnover rate was only 5% compared to the cleavage by FXa, activation occurred at physiological concentrations of prothrombin, which seemed specific since MASP homologues did not mediate this cleavage. Furthermore, MBL-MASP complexes bound to *S. aureus* could generate fibrin that was covalently linked to the bacterial surface via FXIII. This suggests that MBL-MASP may lead to localized fibrin formation. In addition to the action of MASP-2 on coagulation, MASP-1 is also involved in clotting. MASP-1 has thrombin-like activity and activates FXIII and fibrinogen to release fibrinopeptides and to induce clot formation[83]. Although it is tempting to speculate that the two MASPs might act together to cause localized formation of fibrin, a study comparing both actions indicated that clotting via MBL/ficolin-MASP is predominantly catalyzed by MASP-2 cleavage of prothrombin. MASP-1 and MASP-2 can also cleave HK, but release of BK is only shown for MASP-1 [84]. Although the physiological importance of MASPs in coagulation clearly needs further investigations, the fact that these proteases are targeted to bacterial surfaces via pattern recognition molecules might trigger a more specific and localized clotting reaction compared to 'contact' activation.

Vice versa, coagulation proteases can activate the complement system. A number of

coagulation proteases, such as thrombin, FXa, FIXa and FXIa, directly cleave the central complement components C3 and C5 into their bioactive fragments[85]. In addition, it has been shown that the CP can be directly initiated by activation of C1r by FXIIa[86] and that kallikrein has Factor-D-like activity since it converts Factor B to form the AP convertase[87]. Similar to MASP-mediated coagulation activation, the relevance of these findings *in vivo* is presently unknown. However, it is very intriguing that some of these cleavage reactions generate alternate complement activation products that have different biological activities. In particular, thrombin was reported to cleave both the MAC components C5 and C9 into alternative products. First, thrombin cleaves C9 into a 37 kDa C9b molecule[88] that theoretically can cross the peptidoglycan layer. In contrast to native C9, this carboxyl-terminal fragment of C9 is able to disturb the membrane potential of membrane vesicles in absence of C5b-8[89]. Second, thrombin efficiently cleaves C5 at a different cleavage site than the C5 convertase, generating $C5_T$ [90]. This cleavage does not directly lead to release of C5a or C5b, but after cleavage of $C5_T$ by the complement C5 convertase, a different C5b molecule was formed that seemed to form a more potent MAC than the conventional C5b-9 complex. Overall, the crosstalk between complement and coagulation cascades may enhance innate immunity by generating more specific, localized and effective antibacterial reactions.

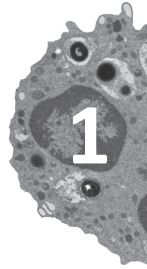
Complement and coagulation in bacterial disease

Whereas complement and coagulation are evolved to act locally at the site of infection and prevent bacterial dissemination, both systems are often pathological during systemic infections. The Systemic Inflammatory Response Syndrome (SIRS), a hallmark of sepsis, is characterized by excessive activation of complement and coagulation[91]. Septic patients have high plasma levels of complement activation products (C3a, C4a and C5a) of which especially C5a is believed to cause an overwhelming inflammatory response that plays a role in the outcome of sepsis. Also coagulation markers like soluble tissue factor and thrombin-antithrombin complexes (TATc) are strongly increased while endogenous inhibitors of coagulation and the fibrinolytic system are consumed[92]. In severe sepsis, coagulation causes disseminated intravascular coagulation (DIC), a serious condition in which microvascular thrombosis, bleeding and microvascular fibrin deposition lead to multiple organ failure, the most common cause of death. Whereas pathogens can directly activate the coagulation system through the contact system, thrombin generation in DIC is mediated by the extrinsic pathway of coagulation[93]. The systemic inflammatory response associated with sepsis disturbs the hemostatic balance, with increased levels of IL-6 leading to tissue factor expression on vascular cells[94] and release of TNFα leading to impaired fibrinolysis. Several experimental studies are now exploring whether therapeutic intervention with complement and coagulation can reduce the mortality of sepsis. Blocking C5a with antibody treatment reduced mortality of experimental models of Gram-negative sepsis (reviewed in [95]). Also in a baboon model of *E. coli* sepsis, complement inhibition at the level of C3 prevented organ damage and pathological coagulation[96]. Still, sepsis is a complex disease and not all forms of sepsis are the same. For instance, whereas C5a has a negative role in Gram-negative sepsis, it seems essential for the immune response during *S. aureus* sepsis[97]. In analogy, inhibition of coagulation via natural anticoagulants has led to contrasting results in the treatment

of sepsis[98]. Thus, even though knowledge of complement and coagulation provides new avenues for the treatment of sepsis, more insights seem required for the pathophysiology of sepsis caused by different bacteria.

Aim of this thesis

Our insights into the molecular interplay between complement, coagulation and bacteria have increased significantly over the past years. The many strategies evolved by pathogenic bacteria, such as *S. aureus*, to modulate complement but also coagulation are strong indicators of a constant battle between bacteria and these protein networks during infection. In this thesis, we aim to obtain a better understanding of these mechanisms and interactions, thereby focusing on the modulation of phagocytosis by *S. aureus*. Furthermore, we investigate mechanisms to enhance phagocytic clearance of staphylococci, which may lead to the development of novel treatment strategies for *S. aureus* infections.



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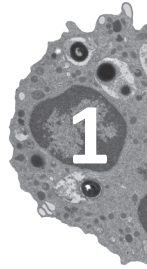

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Chapter 2

Phagocytosis escape by a Staphylococcus aureus protein that connects complement and coagulation proteins at the bacterial surface

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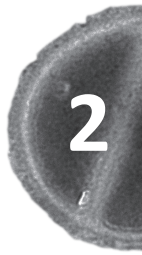
*Equal contribution to manuscript

Abstract

Upon contact with human plasma, bacteria are rapidly recognized by the complement system that labels their surface for uptake and clearance by phagocytic cells. *Staphylococcus aureus* secretes the 16 kD Extracellular fibrinogen binding protein (Efb) that binds two different plasma proteins using separate domains: the Efb N-terminus binds to fibrinogen, while the C-terminus binds complement C3. In this study we show that Efb blocks phagocytosis of *S. aureus* by human neutrophils. *In vitro,* we demonstrate that Efb blocks phagocytosis in plasma and in human whole blood. Using a mouse peritonitis model we show that Efb effectively blocks phagocytosis *in vivo*, either as a purified protein or when produced endogenously by *S. aureus*. Mutational analysis revealed that Efb requires both its fibrinogen and complement binding residues for phagocytic escape. Using confocal and transmission electron microscopy we show that Efb attracts fibrinogen to the surface of complement-labeled *S. aureus* generating a 'capsule'-like shield. This thick layer of fibrinogen shields both surface-bound C3b and antibodies from recognition by phagocytic receptors. This information is critical for future vaccination attempts, since opsonizing antibodies may not function in the presence of Efb. Altogether we discover that Efb from *S. aureus* uniquely escapes phagocytosis by forming a bridge between a complement and coagulation protein.

Author Summary

Staphylococcus aureus is a leading cause of severe bacterial infections in both hospital and community settings. Due to its increasing resistance to antibiotics, development of additional therapeutic strategies like vaccination is required to control this pathogen. Vaccination attempts against *S. aureus* have not been successful so far and an important reason may be the pathogen's elaborate repertoire of molecules that dampen the immune response. These evasion molecules not only suppress natural immunity but also hamper the current attempts to create effective vaccines. In this paper we describe a novel mechanism by which *S. aureus* can prevent uptake by phagocytic immune cells. We discover that the secreted *S. aureus* protein Extracellular fibrinogen binding protein (Efb) generates a 'capsule'-like shield around the bacterial surface through a dual interaction with the plasma proteins complement C3b and fibrinogen. The Efb-dependent fibrinogen shield masks important opsonic molecules like C3b and antibodies from binding to phagocyte receptors. This information is critical for future vaccination attempts, since opsonizing antibodies may not function in the presence of this anti-phagocytic shield.



Introduction

Phagocytosis by neutrophils is crucial to the host innate defense against invading bacteria since it leads to intracellular destruction of bacteria by production of oxygen radicals and proteolytic enzymes[1]. Bacterial engulfment by neutrophils is strongly enhanced by the labeling or 'opsonization' of bacteria with plasma factors such as antibodies and complement activation products (C3b, iC3b)[2]. Complement activation takes place at the bacterial surface and is initiated by recognition molecules (C1q, Mannose Binding Lectin (MBL)) that interact with bacterial surface structures like sugars or proteins[3]. Complement activation occurs through three different pathways (classical, lectin and alternative) that converge in the formation of C3 convertase enzymes that cleave the central complement protein C3[4]. This cleavage step leads to massive decoration of the bacterial surface with covalently deposited C3b and iC3b molecules, which are recognized by complement receptor 1 and 3 (CR1 and CR3) on neutrophils. Complement activation proceeds by formation of C5 convertase enzymes that cleave C5 to release the potent chemoattractant C5a and C5b, which initiates formation of the membrane attack complex[5].

Staphylococcus aureus is an important human pathogen notorious for its ability to cause both community- and hospital-acquired diseases, ranging from mild skin infections to bacteremia, sepsis and endocarditis[6]. Although Methicillin-resistant *S. aureus* (MRSA) was previously considered as an opportunistic pathogen causing hospital-acquired infections in immune-compromised patients, the emergence of the highly virulent community-associated MRSA showed that this bacterium could also cause serious infections in otherwise healthy persons[7]. Due to the rapid emergence of antibiotic resistance strains, alternative therapy options are now being explored[8]. Vaccination has not been successful so far and an important reason may be the bacteria's elaborate immune evasion repertoire. Therefore, immune evasion proteins are now considered as important vaccination targets[9]. One proposed vaccine candidate is Extracellular fibrinogen binding protein (Efb), a 16-kD secreted protein with a presumable role in disease pathogenesis[10,11], which is found in 85% of *S. aureus* strains[12]. Efb consists of two functionally distinct domains: a disordered 9 kD N-terminus (Efb-N) that harbors two binding sites for fibrinogen (Fg)[13] and a folded 7 kD C-terminus (Efb-C) that binds to the C3d domain of complement C3 (which is also present in C3b and iC3b)[14,15]. Although previous papers described various functions for the isolated N- and C-terminal domains of Efb[12–19], it is currently not understood why the full-length Efb protein harbors both a Fg and C3d binding site. In this study we demonstrate that Efb potently blocks phagocytosis of bacteria via a novel mechanism linking the complement and coagulation proteins.

Results

Full-length Efb inhibits phagocytosis in the presence of plasma

To study a potential role for full-length Efb in phagocyte escape, we mixed fluorescently labeled *S. aureus* with purified human neutrophils, Efb (0.5 μM) and human serum or plasma as a source for complement and analyzed bacterial uptake by flow cytometry. In the presence of serum, Efb did not affect bacterial uptake by neutrophils (**figure 1A**). However when we used human plasma as a complement source, we found that Efb strongly prevented phagocytosis (**figure 1A,B**) and subsequent bacterial killing by neutrophils (**supplemental figure 1**). Phagocytosis inhibition in plasma occurred in a dose-dependent fashion with a calculated IC_{50} of 0.08 μM (**figure 1C**). Since the main difference between plasma and serum lies in the presence of coagulation proteins, we investigated whether the observed differences in phagocytosis inhibition were caused by the fact that serum lacks Fg. Indeed, we observed that supplementation of serum with physiological concentrations of Fg led to phagocytosis inhibition by Efb (**figure 1D**). Fg is a large (340 kD) dimeric protein that comprises one central E-fragment and two lateral D-fragments. Since Efb binds to the D-fragment of Fg[13], we examined if supplementing serum with Fg-D would also lead to phagocytosis inhibition by Efb. Interestingly, we found that Efb could not block phagocytosis in the presence of Fg-D (**figure 1E**) indicating that full-length Fg is required for phagocytosis inhibition by Efb. Since Fg is a ligand for CR3 (or Mac-1 [20]) on neutrophils, we studied whether the binding of Fg to this receptor is important for the anti-phagocytic effect of Efb. Therefore, we purified Fg from wild-type mice or $Fg\gamma^{390–396A}$ (ΔMac-1 Fg) mice that express a mutated form of Fg lacking the Mac-1 binding site but retaining clotting function[21]. **Figure 1F** shows that supplementation of human serum with both forms of mouse Fg led to inhibition by Efb, indicating that Fg binding to Mac-1 is not important for inhibition. In conclusion, Efb interferes with phagocytosis in a plasma environment and the presence of full-length Fg is required for this inhibition.

Simultaneous binding to Fg and C3 is essential for phagocytosis inhibition by Efb

To get more insight into the mechanism of inhibition, we constructed a panel of Efb mutants (**figure 2A**). First we observed that the individual N- or C-termini of Efb could not block phagocytosis in plasma (**figure 2B**). In addition, mixing the N- and C-terminal fragments of Efb did not markedly affect phagocytosis, indicating that full-length Efb is required. Second, we generated mutants of full-length Efb lacking the previously characterized binding sites for Fg and C3 (**figure 2A** [13,14]). We created three different Fg-binding mutants: EfbΔFg1 lacking residues 30-45, EfbΔFg2 lacking residues 68-76 and EfbΔFg1+2 lacking both these Fg binding sites. Furthermore we created EfbΔC3 in which the C3d-binding residues R131 and N138 were each replaced with a glutamic acid (E) (also known as Efb-RENE[14]). Using direct binding ELISAs we verified that EfbΔFg1+2 could no longer bind Fg, while the single EfbΔFg1 and EfbΔFg2 mutants and EfbΔC3 still bound Fg (**supplemental figure 2A**). As expected, all mutants except EfbΔC3 bound to C3b (**supplemental figure 2B**). Next, we compared these mutants in the neutrophil phagocytosis assay in the presence of human plasma. We show that EfbΔFg1+2 and EfbΔC3 could no longer block phagocytosis (**figure 2C**), indicating that

a simultaneous interaction with both Fg and complement C3 (products) is essential for the anti-phagocytic action of Efb. The finding that EfbΔFg1 and EfbΔFg2 were still active indicates that Efb requires only one of its two Fg binding sites to block phagocytosis.

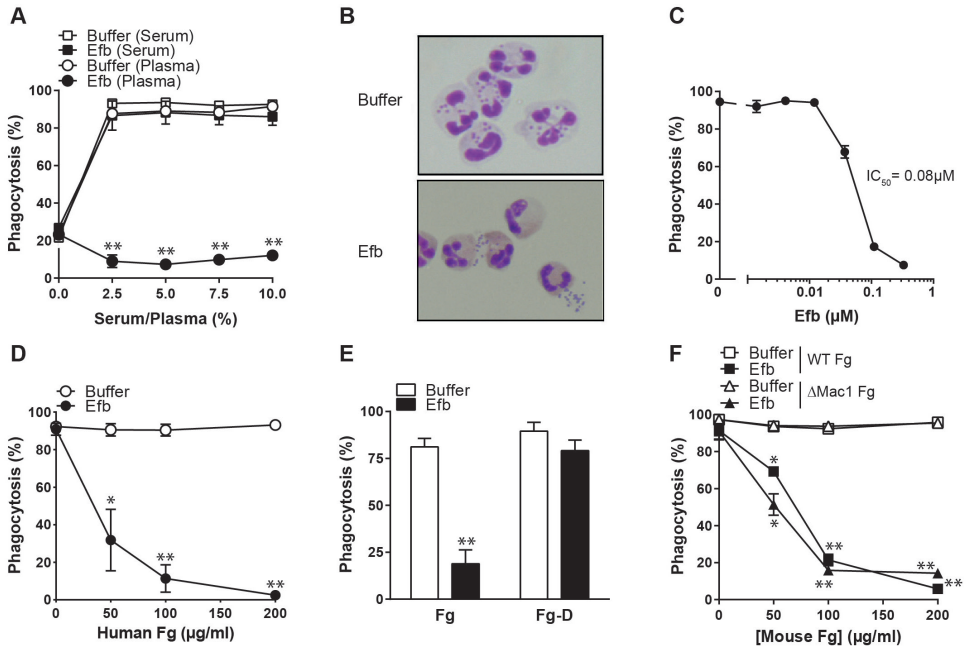


Figure 1. Full-length Efb inhibits phagocytosis of *S. aureus* in human plasma. A. Phagocytosis of fluorescently labeled *S. aureus* by purified human neutrophils in the presence of human serum or plasma and Efb (0.5 µM). **B.** Histology image of human neutrophils incubated with *S. aureus* and 2.5% plasma in the presence or absence of Efb (0.5 µM). Cells were stained using Diff-Quick. **C.** Dose-dependent phagocytosis inhibition by Efb in the presence of 2.5% human plasma. IC_{50} was calculated using non-linear regression analysis, R^2=0.95. **D-F.** Phagocytosis in the presence of 5% human serum supplemented with either full-length human Fg (**Fig. 1D**), the D domain of human Fg (1 µM or 86 µg/ml) (**Fig. 1E**) or mouse Fg (WT or lacking the Mac-1 binding site) (**Fig. 1F**). **A, C-F** are mean ± se of three independent experiments. B is a representative image. **P*<0.05, ***P*<0.005 for Efb versus buffer (two-tailed Student's *t*-test).

Efb blocks phagocytosis *ex vivo* and *in vivo*

To study whether Efb can also block phagocytosis in a natural environment, we tested its activity in *ex vivo* and *in vivo* phagocytosis models. In an *ex vivo* human whole blood model, we incubated fluorescent *S. aureus* with 50% human whole blood and Efb. After 25 minutes, neutrophil phagocytosis was analyzed by flow cytometry. We observed that full-length Efb potently blocked phagocytosis by human neutrophils in whole blood (**figure 3A**) and that this inhibition depends on the interaction of Efb with both Fg and C3. Next, we studied phagocytosis of *S. aureus* in an *in vivo* mouse peritonitis model. To this end, mice were treated with carrageenan intraperitoneally (i.p.) to induce neutrophil infiltration into the peritoneal cavity and subsequently challenged with 10^8 heat-inactivated fluorescent *S. aureus* in the presence or absence of Efb (1 µM). One hour later, mice were sacrificed and the peritoneum was lavaged with sterile PBS. Neutrophils were stained and phagocytosis of fluorescent bacteria was analyzed by flow cytometry. We observed that Efb blocked phagocytosis in the

peritoneum (**figure 3B,C**). Efb mutants showed that inhibition of phagocytosis *in vivo* also depends on the Fg and C3 binding domains of Efb.

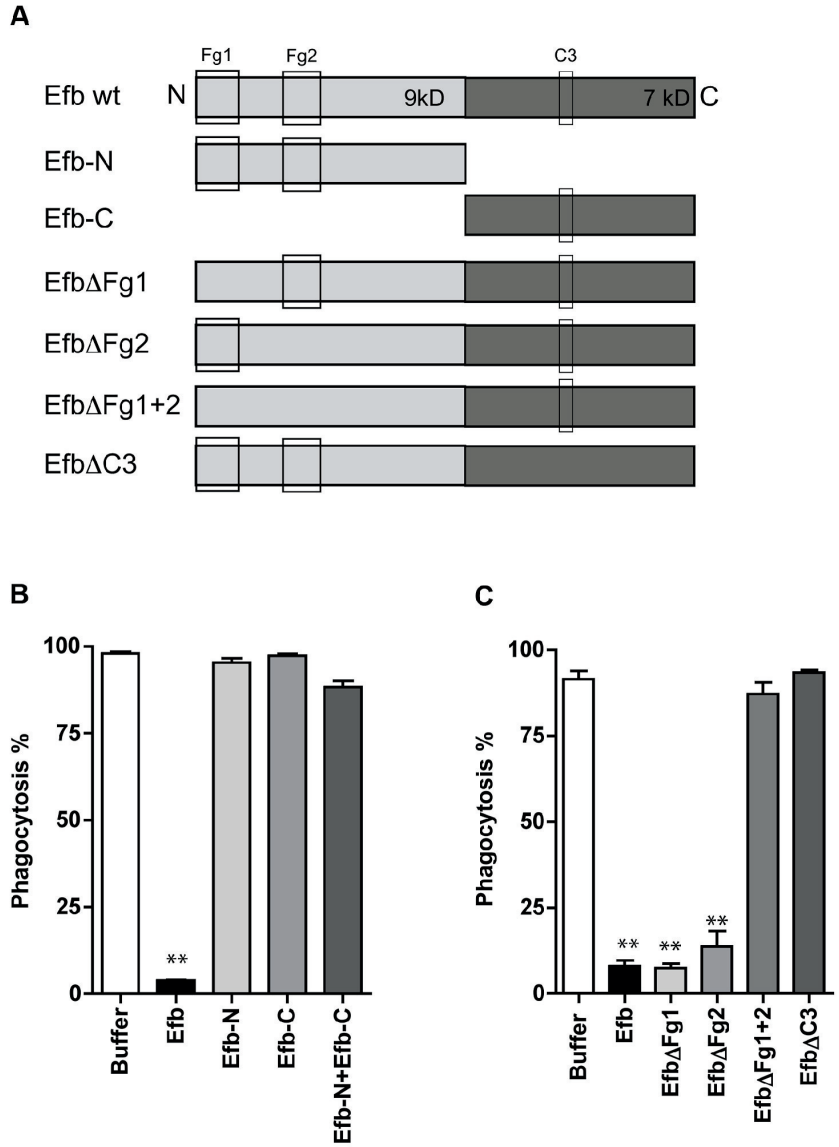


Figure 2. Simultaneous binding to Fg and C3 is essential for phagocytosis inhibition by Efb. A. Schematic overview of Efb mutants generated in this study. Efb is depicted in its secreted form (30-165) lacking the signal peptide (1-29). Bounding boxes indicate Fg- and C3-binding domains. The N-terminus of Efb (light grey, 9 kD) harbors two Fg binding sites named Fg1 (residues 30-67) and Fg2 (residues 68-98). The C-terminus of Efb (dark grey, 7 kD) harbors the C3 binding site (residues R131 and N138). EfbΔFg1 has deletion of residues 30-45, resulting in non-functional binding Fg1; whereas EfbΔFg2 has deletion of residues 68-76, resulting in non-functional binding Fg2. **B-C.** Phagocytosis of fluorescent *S. aureus* by human neutrophils in the presence of 5% human plasma and Efb fragments (**B**) or Efb mutants (**C**) (all at 1 µM). **B,C** are mean ± se of three independent experiments. ***P*<0.005 for Efb versus buffer (two-tailed Student's *t*-test).

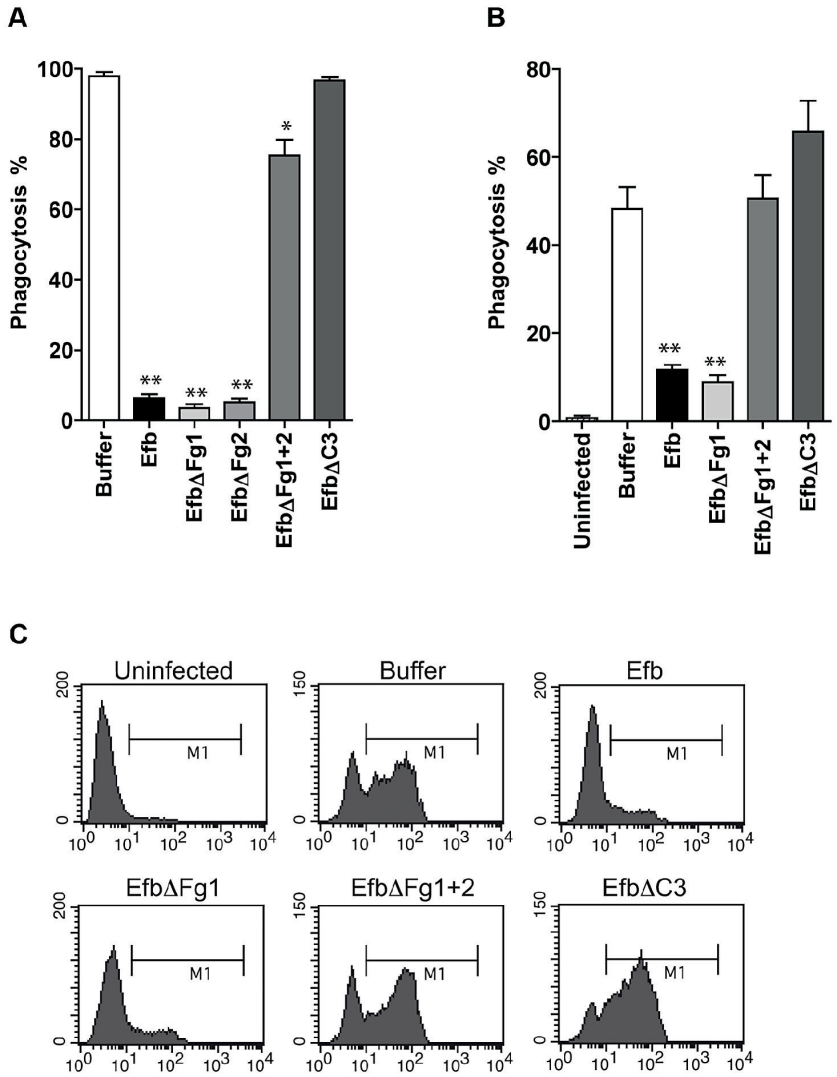


Figure 3. Purified Efb blocks phagocytosis *ex vivo* and *in vivo*. **A.** *Ex vivo* phagocytosis of fluorescent *S. aureus* incubated with 50% human whole blood and Efb (1 μM). Neutrophils were gated based on forward and side scatter properties. **B.** *In vivo* phagocytosis of fluorescent *S. aureus* by human neutrophils in the mouse peritoneum. Neutrophils were attracted to the peritoneal cavity using carrageenan (i.p.) and subsequently challenged with 10^8 heat-inactivated fluorescent *S. aureus* and Efb (1 μM) for 1 hour. The peritoneal lavage was collected and neutrophil phagocytosis was analyzed by flow cytometry. Neutrophils were gated based on Gr-1 expression. The mouse experiments were carried out three times. In each experiment, we used 3 mice per group and the cells of these 3 mice were pooled for phagocytosis analysis. **C.** Representative histograms of **B. A,B** are mean ± se of three independent experiments. *P<0.05, **P<0.005 for Efb versus buffer (two-tailed Student's t-test).

Phagocytosis inhibition by Efb is independent of complement inhibition

Experiments shown above indicate that Efb requires an interaction with both complement

and Fg to block phagocytosis. To study whether Efb also interacts with *S. aureus* specifically, we analyzed whether purified Efb can block phagocytosis of other bacteria as well. Fluorescent *S. epidermidis* or *E. coli* were mixed with human plasma and phagocytosis by neutrophils was evaluated. Efb potently inhibits the uptake of these bacteria as well, indicating that Efb can block phagocytosis independently of *S. aureus* (**figure 4A**). Previously, we observed that the C-terminal domain of Efb is a complement inhibitor that inactivates C5 convertases to prevent cleavage of C5[12]. Efb-C did not affect C3b labeling of bacteria in conditions where all complement pathways are active. However, since the effects of Efb on complement inhibition were performed with serum instead of plasma, we wondered whether full-length Efb might affect C3b labeling of bacteria in a plasma environment. Therefore, we incubated *S. aureus* with human plasma and Efb and quantified surface-bound C3b using immunoblotting. As a control, we added EDTA to prevent activation of all complement routes (which are calcium and magnesium dependent). Similar amounts of C3b were found on the bacterial surface in the presence of Efb compared to buffer (**figure 4B**), indicating that Efb does not interfere with C3b labeling in plasma. Subsequently, we re-analyzed the inhibition of C5 convertases by Efb (mutants) in plasma using an alternative pathway hemolytic assay. Rabbit erythrocytes were incubated with human plasma and C5 cleavage was measured by means of C5b-9 dependent lysis of erythrocytes. In conjunction with previous results in serum, we found that all Efb mutants except for EfbΔC3 inhibited C5 cleavage in plasma (**figure 4C**). Since this inhibition exclusively depends on the C-terminal domain (all Fg binding mutants of Efb could still block C5 cleavage), this proves that interference with C5 cleavage is at least not sufficient for phagocytosis inhibition by Efb. To further show that the effects of Efb on complement activation are dispensable for phagocytosis inhibition, we introduced a washing step in our phagocytosis assay. Bacteria were first incubated with serum (in the absence of Efb) to deposit C3b. After washing away unbound serum proteins (including C5a), these pre-opsonized bacteria were incubated with Fg and neutrophils. In this assay, Efb could potently block phagocytosis (**figure 4D**). In conclusion, these results indicate that the anti-phagocytic activity of Efb is not related to its complement-inhibitory effect.

Efb covers *S. aureus* with a shield of Fg

We wondered whether Efb might bind to C3b-labeled bacteria and then attract Fg to the surface. First, we studied whether full-length Efb can bind to Fg and C3b at the same time. C3b-coated microtiter plates were incubated with Efb and, after a washing step, treated with Fg. **Figure 5A** shows that Efb is able to form a complex with C3b and Fg. Also, the EfbΔFg1 and EfbΔFg2 mutants could still form Fg-C3b complexes. In contrast, complex formation was not detected for the mutants that lack either both Fg (EfbΔFg1+2) or the C3 binding domains (EfbΔC3) (**figure 5A**). Then, we investigated whether Efb could attract Fg to pre-opsonized bacteria. Therefore, *S. aureus* was pre-opsonized with human serum to deposit complement and subsequently incubated with Efb. After washing, bacteria were incubated with Alexa-488 conjugated Fg. Using both flow cytometry and confocal microscopy we observed that Efb mediates Fg binding to pre-opsonized bacteria (**figure 5B,C**). Consistent with the ELISA data for complex formation, no Fg binding was detected in the presence of EfbΔFg1+2 or EfbΔC3. Confocal analyses indicated that Efb covers the complete bacterial

surface with Fg (**figure 5C**). Using Transmission Electron Microscopy we analyzed this Fg layer created by Efb in more detail. After incubation of *S. aureus* with plasma and Efb, we observed a diffuse outer layer formed around the bacteria (**figure 5D**). Altogether these experiments show that Efb binds to C3b on the bacterial surface and subsequently attracts Fg forming a shield around the bacterial surface.

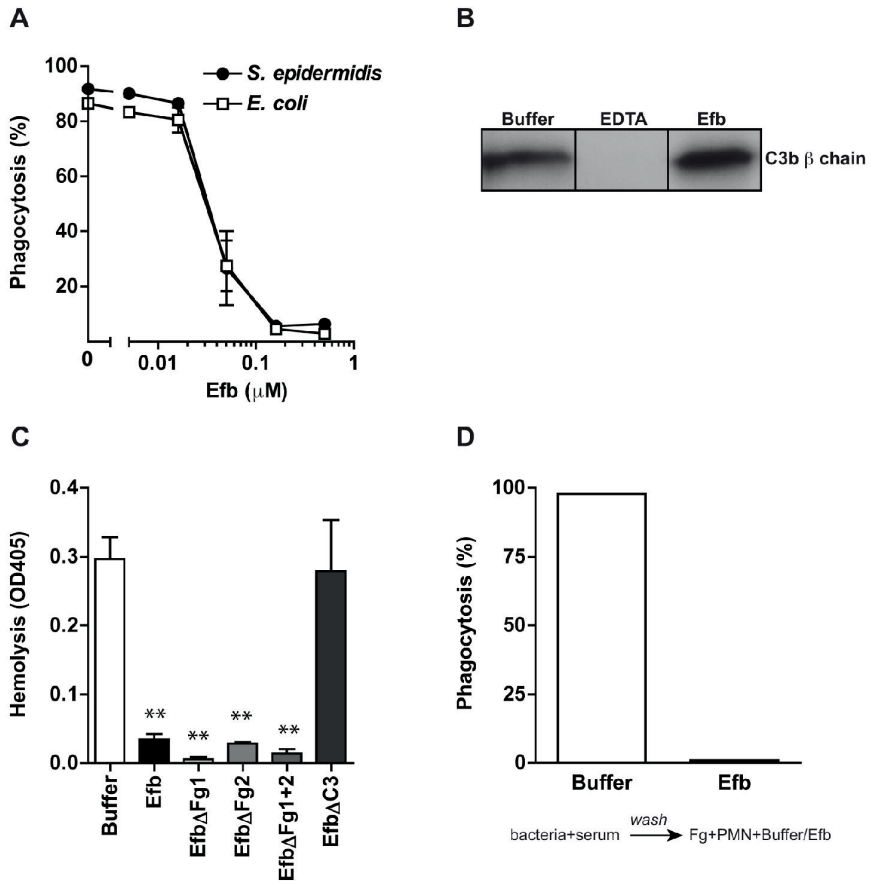


Figure 4. Phagocytosis inhibition by Efb is independent of complement inhibition. A. Phagocytosis of fluorescently labeled *S. epidermidis* and *E. coli* by purified human neutrophils in the presence of human plasma (5%) and Efb. **B.** Immunoblot detecting surface-bound C3b after incubation of *S. aureus* with 5% human plasma in the presence of 5 mM EDTA or 0.5 μM Efb. Blot is a representative of 3 independent experiments. **C.** Alternative pathway hemolysis of rabbit erythrocytes in 5% human plasma and Efb (mutants) (1 μM). Bars are the mean ± se of three independent experiments. $^{**}P<0.005$ for Efb versus buffer (two-tailed Student's *t*-test). **D.** Phagocytosis with a washing step. Fluorescent *S. aureus* was first incubated with 5% serum to deposit complement. Bacteria were washed and subsequently mixed with neutrophils and Fg in the presence or absence of Efb (0.5 μM). Graph is a representative of three independent experiments.

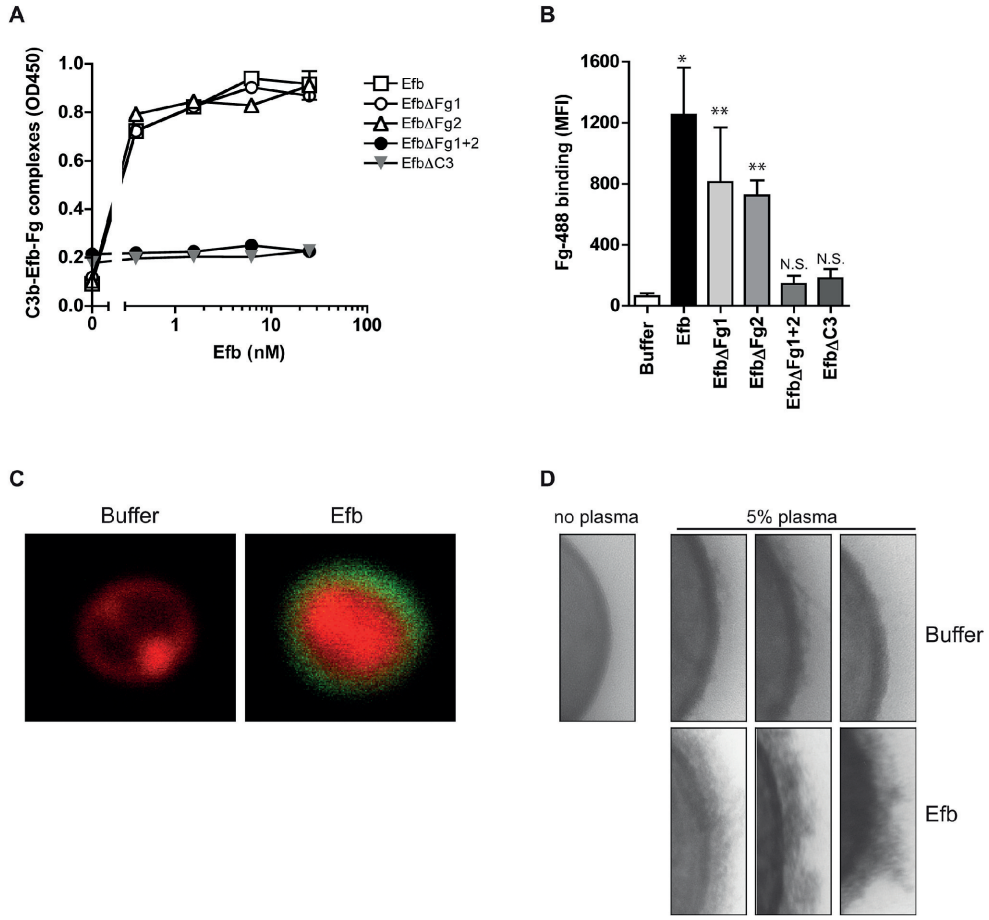


Figure 5. Efb attracts Fg to the bacterial surface. A. ELISA showing that Efb can bind Fg and C3b at the same time. C3b-coated microtiter wells were incubated with Efb (mutants) and, after washing, incubated with 50 nM Fg that was detected with a peroxidase-conjugated anti-Fg antibody (Abcam). Graph is a representative of two independent experiments performed in duplicate. **B.** Binding of Alexa488-labeled Fg (60 μg/ml) to serum-opsonized *S. aureus* in the presence of Efb (mutants) (0.5 μM). Graph represents mean ± se of three independent experiments. $^{*}P<0.05$, $^{**}P<0.005$ for Efb versus buffer (two-tailed Student's *t*-test). N.S. is not significant. **C.** Confocal analysis of samples generated in B (representative images). **D.** TEM pictures of *S. aureus* incubated with 5% human plasma in the absence or presence of Efb (0.5 μM). Three representative images are shown.

Efb blocks recognition of C3b and IgG on the surface

Since Efb covers bacteria with a shield of Fg, we hypothesized that this would frustrate the binding of phagocytic receptors to their ligands on the bacterial surface Using flow cytometry, we first analyzed whether C3b-labeled bacteria were still recognized by CR1. Pre-opsonized *S. aureus* was incubated with soluble CR1 in the presence of Fg and Efb. Clearly, binding of CR1 to pre-opsonized bacteria was blocked by the presence of both Fg and Efb (**figure 6A**). Addition of Fg or Efb alone did not affect CR1 binding. Next, we investigated whether the Fg shield specifically blocks C3b-CR1 interactions or whether it

also disturbs the binding of neutrophil Fc receptors to opsonic antibodies. To analyze this, we determined whether the Fc part of bacterium-bound IgG could still be recognized by specific antibodies. We found that incubation of pre-opsonized bacteria with Efb and Fg disturbs recognition of the antibody Fc domain on the surface (**figure 6B**), suggesting that Fc receptors can no longer recognize their target. This information is crucial for future vaccine development since opsonic antibodies against *S. aureus* may not function when Efb hides these antibodies underneath an Fg shield. To further prove that Efb functionally blocks opsonization, we analyzed phagocytosis of an encapsulated *S. aureus* strain in the presence or absence of anti-capsular antibodies. The encapsulated *S. aureus* strain Reynolds was grown for 24 hours in Columbia agar supplemented with 2% NaCl (for optimal capsule expression[22]) and subsequently labeled with FITC. We verified capsule expression after FITC-labeling using specific antibodies (**supplemental figure 3**). In low plasma concentrations (0-1%), we observed that anti-capsular antibodies caused a 6-fold increase in phagocytic uptake of encapsulated *S. aureus* (**figure 6C**). At these plasma concentrations, Efb could not block phagocytosis. However at higher plasma concentrations (3% and more), Efb potently impeded phagocytosis in the presence of anti-capsule antibody (**figure 6C**). These data support our idea that the Fg shield created by Efb prevents recognition of important opsonins like C3b and IgG, also in the context of a capsule-expressing strain that is targeted by specific antibodies.

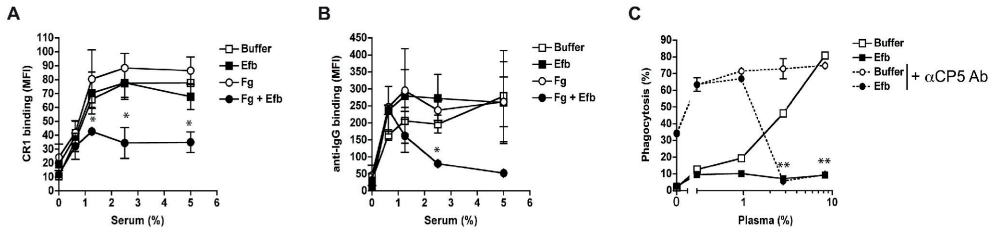


Figure 6. Efb prevents recognition of opsonic C3b and IgG. A-B. Flow cytometry assay detecting binding of soluble CR1 (**A**) or anti-IgG antibody (**B**) to pre-opsonized *S. aureus* in the presence of buffer, Efb (0.5 μM) and/or Fg (200 μg/ml). **C.** Efb inhibits phagocytosis of encapsulated *S. aureus* by human neutrophils. FITC-labeled *S. aureus* strain Reynolds (high capsule CP5 expressing strain) was incubated with human plasma and/or Efb (0.5 μM) in the presence (dotted line) or absence (solid line) of polyclonal rabbit anti-CP5 antibody. All figures represent the mean ± se of three separate experiments. **P*<0.05, ***P*<0.005 for Efb+Fg versus buffer (**A,B**) or Efb versus buffer (for dotted lines) (two-tailed Student's *t*-test).

Endogenous Efb blocks phagocytosis *in vitro* and *in vivo*

To study whether endogenous expression of Efb leads to impaired phagocytosis of *S. aureus* via complex formation, we extended our analyses with (supernatants of) an isogenic Efb-deletion mutant in *S. aureus* Newman (previously described in [23]). First we performed Immunoblotting to semi-quantify the production levels of Efb in liquid bacterial culture supernatants. Supernatants of wild-type (WT) *S. aureus* Newman were subjected to Immunoblotting and developed using polyclonal anti-Efb antibodies (**figure 7A**). Efb expression in the supernatant was quantified using ImageJ software and compared with fixed concentrations of purified (His-tagged) Efb using linear regression analysis (R^2=0.986). Efb levels in 4h and 20h supernatants contained 1,1 μM and 0,9 μM Efb respectively.

Although the Efb levels in strain Newman are suspected to be higher than in other *S. aureus* strains (up to 10-fold[24], due to a point mutation in the SaeR/S regulatory system that drives expression of immune evasion genes[25]), the fact that these levels are >10 times higher than the calculated IC_{50} needed for phagocytosis inhibition (0.08 μM, **figure 1C**), suggests that Efb concentrations required for phagocytosis inhibition can be reached *in vivo*. In a separate Immunoblot, we checked for the presence of Efb in 4h supernatants of the WT, Efb-deficient (ΔEfb) and the complemented strain (ΔEfb+pEfb) confirming the lack of Efb expression in the mutant (**figure 7A**). Next we used these supernatants to study whether endogenous Efb can mediate C3b-Fg complex formation on the bacterial surface. *S. aureus* was first incubated with serum to deposit C3b, then mixed with bacterial supernatants and subsequently incubated with fluorescently labeled Fg. Whereas WT supernatants attracted Fg to the surface of pre-opsonized bacteria, Efb-deficient supernatants did not mediate complex formation (**figure 7B**). This phenotype was restored in the complemented strain. Then we studied whether endogenous Efb could inhibit phagocytosis by neutrophils *in vitro.* Therefore we repeated the latter experiment (but using fluorescent bacteria and unlabeled Fg) and subsequently mixed the bacteria with human neutrophils. We found that supernatants of WT and complemented strains inhibited phagocytosis, while Efb-deficient supernatants did not influence this process (**figure 7B**).

To mimic bacterial phagocytosis during a natural infection, carrageenan-treated mice were injected i.p. with GFP-expressing WT *S. aureus* or the Efb-deficient mutant in their original broth culture and sacrificed 1h thereafter. Mice were subjected to peritoneal lavage and the percentage of neutrophils with internalized staphylococci was determined by flow cytometry. As depicted in **figure 7D**, the Efb-deficient *S. aureus* strain was phagocytosed by neutrophils to a significantly higher extent than the WT strain despite of the fact that the amount of inoculated bacteria was comparable in both groups (app. $2x10^7$). These observations demonstrate that the levels of Efb produced by *S. aureus* are sufficient for preventing phagocytosis *in vivo*.

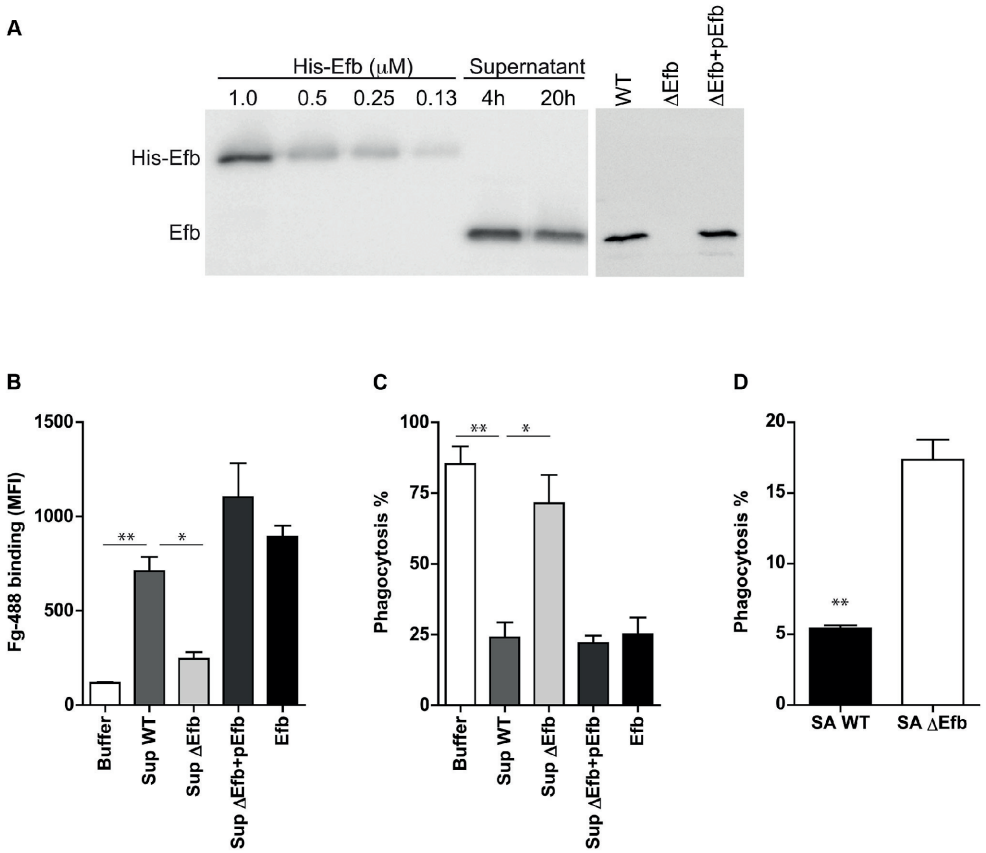


Figure 7. Endogenously produced Efb blocks phagocytosis via complex formation. A. *Left.* Immunoblot detecting Efb in 4h and 20h culture supernatants of *S. aureus* Newman; fixed concentrations of His-tagged Efb were loaded as controls. *Right.* Immunoblot of 4h culture supernatants of *S. aureus* Newman (WT), an isogenic Efb deletion mutant (ΔEfb) and its complemented strain (ΔEfb+pEfb). Blots were developed using polyclonal sheep anti-Efb and Peroxidase-labeled donkey anti-sheep antibodies. Blot is a representative of two independent experiments. **B.** Flow cytometry analysis of the binding of Alexa488-labeled Fg to pre-opsonized *S. aureus* in the presence of 4h culture supernatants (2-fold diluted) or purified Efb (250 nM). **C.** *In vitro* phagocytosis of fluorescently labeled *S. aureus* by purified human neutrophils. Pre-opsonized *S. aureus* was first incubated with 4h culture supernatants (2-fold diluted) or purified Efb (250 nM) and subsequently mixed with Fg and neutrophils. **D.** *In vivo* phagocytosis of GFP-expressing wild-type or Efb-deficient *S. aureus* strains by neutrophils in the mouse peritoneal cavity. Neutrophils were attracted to the peritoneal cavity using carrageenan (i.p.) and subsequently injected with 300 µl of GFP-expressing wild-type (SA WT) or Efb-deficient (SAΔEfb) *S. aureus* strains during the exponential phase of growth. The peritoneal lavage was collected 1h thereafter and neutrophil phagocytosis was analyzed by flow cytometry. Neutrophils were gated based on Gr-1 expression. Graphs in **B-D** represent mean ± se of three independent experiments. **P*<0.05, ***P*<0.005 for WT versus Buffer or ΔEfb (two-tailed Student's *t*-test).

Discussion

The coagulation system has a dual role in the host defense against bacterial infections. On one hand, coagulation supports innate defenses by entrapment and killing of invading bacteria inside clots[26] or via the formation of small antibacterial and pro-inflammatory peptides[2727,28]. On the other hand, bacterial pathogens can utilize coagulation proteins to protect themselves from immune defenses. In this study, we find that *S. aureus* effectively protects itself from immune recognition by secreting Efb that specifically attracts Fg from the solution to the bacterial surface creating a capsule-like shield (**figure 8**). To accomplish this, Efb forms a multi-molecular complex of soluble Fg and surface-bound C3b. The fact that the levels of C3b at the bacterial surface are high[29] and that Fg is an abundant plasma protein (1.5-4.0 g/L) makes this a very efficient anti-phagocytic mechanism. The Fg shield created by Efb effectively protects *S. aureus* from recognition by phagocyte receptors. We found that the attracted Fg does not only block the binding of C3b to its receptor, but also hides the important opsonin IgG underneath the Fg shield. We think that this information is critical for future vaccine development against *S. aureus*. Generation of protective 'opsonizing' antibodies recognizing *S. aureus* surface structures is considered to be an important goal of vaccination. However, these antibodies will not function if they are protected underneath a layer of Fg. We anticipate that including Efb in future vaccines might be beneficial as it could prevent formation of this anti-phagocytic shield and enhance the function of opsonizing antibodies. The fact that Efb is conserved among *S. aureus* strains may make it a suitable vaccine candidate[30].

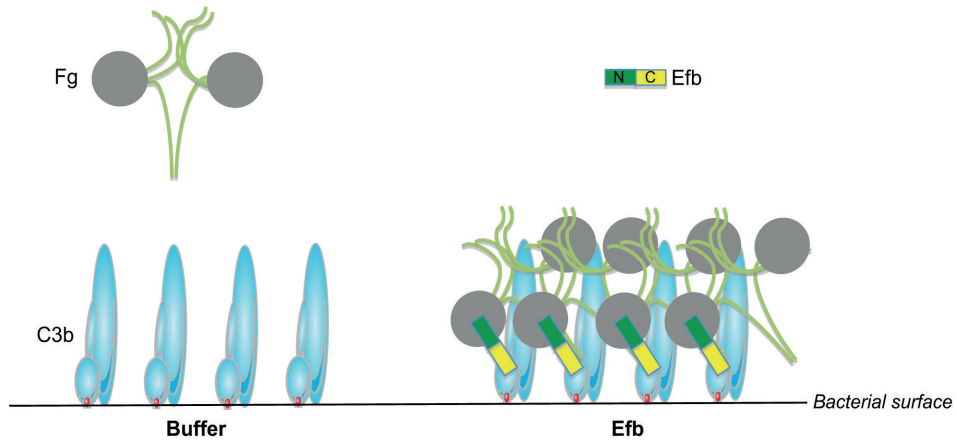


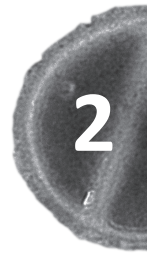
Figure 8. Proposed mechanism for phagocytosis inhibition by Efb. Schematic picture of the proposed phagocytosis escape mechanism by Efb. *Left*, Complement activation on the bacterial surface results in massive labeling of *S. aureus* with C3b molecules while Fg stays in solution. *Right*, *S. aureus* secretes Efb, which binds to surface-bound C3b via its C-terminal domain (colored yellow). Using its N-terminus (green), Efb attracts Fg to the bacterial surface. This way, *S. aureus* is covered with a shield of Fg that prevents binding of phagocytic receptors to important opsonins like C3b and IgG.

Next to Efb, *S. aureus* secretes two other proteins that specifically interact with the coagulation system: the *S. aureus* 'coagulases' named Coagulase and Von Willebrand factor binding protein are secreted proteins that activate prothrombin in a nonproteolytic manner and subsequently convert Fg into fibrin[31]. Thereby, coagulases embed bacteria within a network of fibrin, protecting them from immune recognition and facilitate formation of *S. aureus* abscesses and persistence in host tissues[32]. Coagulase and Efb are expressed at the same time during infection since they are both regulated by the SaeR/S regulator for secreted (immune evasion) proteins[25,33]. Based on our study, we hypothesize that Efb may be highly important for proper functioning of Coagulase since Efb can attract Fg to the bacterial surface. This way, Efb may aid Coagulase-dependent fibrin formation to occur close to the bacterial surface instead of in solution. Nevertheless our studies also indicate that Efb can block phagocytosis in the absence of prothrombin and Coagulase. However, in a more complex environment the anti-phagocytic mechanisms of Efb and *S. aureus* Coagulase might work synergistically. Furthermore, it seems tempting to speculate that the ability of Efb to attract Fg to the bacterial surface is also beneficial in other infection processes like adhesion. Since Fg is an important constituent of the extracellular matrix (ECM), Efb might also facilitate binding of C3b-opsonized bacteria to the ECM. In fact, Efb was previously classified as an adhesion molecule belonging to the group of SERAMs (secreted expanded repertoire adhesive molecules)[34]. However, as a secreted protein, Efb cannot facilitate bacterial adhesion if it solely binds to Fg in the ECM without interacting with the bacterial surface. Binding to C3b-labeled bacteria via the Efb C-terminus might therefore be crucial for effective bacterial adhesion to Fg.

The pathogenic potential of *S. aureus* is a result of its versatile interactions with multiple host factors, evidenced by the fact that it can survive at multiple sites of the body causing a wide range of infections. At most body sites, *S. aureus* has to deal with cellular and humoral components of the immune system. However, increasing evidence now suggests that *S. aureus* protects itself from immune defense by forming abscess communities surrounded by capsule-like structures that prevent neutrophil invasion[35,36]. Our study implicates that Efb might be crucial in the formation of these capsules. Furthermore, our whole blood assays shows that Efb may also play an important role in *S. aureus* survival in the blood allowing it to spread to other sites of the body. Previous studies using animal models have highlighted the critical role of Efb in *S. aureus* pathogenesis. For instance, Efb delays wound healing in a rat wound infection model[10] and is important for *S. aureus* pneumonia and abscess formation in kidneys[23]. Our *in vivo* studies corroborate the *in vitro* findings and suggest that complex formation can occur under physiological conditions *in vivo*. However, the available mouse models do not closely mimic this process during clinical infections in humans. Efb is produced in later stages of bacterial growth, thus the bacteria need time to produce Efb before they come into contact with neutrophils. Since neutrophils need to be recruited from the blood to the site of the infection, there normally is time for Efb production and complex formation, especially in the human host where an infection starts with a low number of bacteria. In contrast, in available mouse models the timing is much different as a high inoculum (up to 10^8 bacteria) is required to establish an infection and these high numbers of bacteria trigger a strong inflammatory response resulting in that the bacteria are already

phagocytized before Efb is produced. For this reason, we have mixed the bacteria with their supernatants to ensure the presence of endogenous Efb during the course of the experiments and have chosen a model in which neutrophils are already attracted to the infection site to focus on the anti-phagocytic activity of the molecule. Future studies are needed to design and execute appropriate animal studies that overcome the limitations of current models and better reflect the clinical situation.

In summary, we describe that full-length Efb can inhibit phagocytosis in a unique way through its dual interaction with complement and Fg. Our study indicates that Efb is a highly effective immune escape molecule that blocks phagocytosis of *S. aureus in vivo*.



Materials & Methods

Ethics statement

Study participants provided written informed consent in accordance with the Declaration of Helsinki. Approval was obtained from the medical ethics committee of the UMC Utrecht. Animal experiments were performed in strict accordance with the German regulations of the Society for Laboratory Animal Science (GV-SOLAS) and the European Health Law of the Federation of Laboratory Animal Science Associations (FELASA). All experiments were approved by the ethical board Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg in Germany (Permit No. 33.9-42502-04-10/0296).

Bacterial strains, fluorescent labeling and supernatants

In this study we used the laboratory *S. aureus* strains Newman, SH1000, Reynolds and Wood 46 (with low expression of Protein A). The *S. aureus* strain KV27 and the *S. epidermidis* and *E. coli* strains were clinical isolates obtained within the UMCU. Targeted deletion (and complementation) of Efb in *S. aureus* Newman was described previously in[23]. All strains were cultured overnight on Tryptic Soy Blood Agar (BD) or Todd Hewitt Agar (with appropriate antibiotics) at 37°C. The capsule-expressing *S. aureus* strain Reynolds and its isogenic CP5-deficient mutant were a kind gift from Jean Lee (Harvard Medical School, Boston, USA)[22]. To optimize capsule expression, strain Reynolds was grown on Columbia Agar supplemented with 2% NaCl (CSA) for 24h at 37°C. For fluorescent labeling of strains, bacteria were resuspended in PBS and incubated with 0.5 mg/ml FITC (Sigma) for 30 minutes on ice. Bacteria were washed twice with PBS, resuspended in RPMI medium with HSA and stored at -20°C until further use. For *in vivo* experiments, *S. aureus* Newman and the Efb mutant were transformed with the pCM29 plasmid (kindly provided by Alexander Horswill, University of Iowa) allowing constitutive expression of the superfolder green fluorescent protein (sGFP) via the sarAP1 promoter[37]. To isolate bacterial supernatants, WT and mutant strains were cultured overnight in Todd Hewitt Broth (THB) without antibiotics and subsequently sub cultured in fresh THB for 4h or 20h. Cultures were centrifuged at 13,000 rpm and collected supernatants were stored at -20°C until further use.

Protein expression and purification

Recombinant Efb proteins were generated in *E. coli* as described previously[12,13]. Briefly, (parts of) the *efb* gene from *S. aureus* strain Newman (without the signal peptide) were amplified by PCR and ligated into either the pGEX-5x-1 vector (GE healthcare) or the pRSETB vector (Invitrogen) for N-terminal fusions with glutathione S-transferase (GST) or polyhistidine respectively. Mutations of the Fg and C3 binding domains were introduced in pGEX plasmids containing full-length GST-Efb as described previously[13]. Recombinant proteins were expressed and purified according to the manufacturer's manual. In all experiments where wild-type Efb was compared with mutants, we used GST-tagged Efb. Otherwise His-tagged Efb was used.

ELISA

Microtiter plates were coated with human C3b[38] or Fg, blocked with 3% BSA-PBS, and incubated with 6 nM Efb for one hour at room temperature. Efb binding was detected using peroxidase-conjugated rabbit anti-GST polyclonal antibodies (Abcam) and quantified using *O*-phenylenediamine dihydrochloride (Sigma). To study formation of C3b-Efb-Fg complexes, C3b-coated plates were incubated with Efb for one hour at room temperature. After washing, human Fg (50 nM) was added and detected through incubation with peroxidase-conjugated anti-Fg antibodies (Abcam).

Preparation of Fg-D fragments

D fragments of Fg were generated by digestion of human Fg (Enzyme research) with plasmin (Enzyme research, 10 µg/15 mg Fg) in TBS containing 10 mM $CaCl_2$ for 4 hours at 37ºC as described earlier[39] with modifications. D fragments (85 kD) were purified by gel filtration on Sephacryl S-200 (GE Healthcare) and analyzed by SDS-PAGE.

Purification of human blood products

For preparation of plasma, venous blood from 10 healthy volunteers was collected in glass vacutainers (BD) containing the anticoagulant lepirudin (50 µg/ml)[40]. To prepare serum, blood was collected in glass vacutainers (BD) without anticoagulant and allowed to clot for 15 min at room temperature. Plasma and serum were collected after centrifugation for 10 minutes at 4000 rpm at 4°C, pooled and subsequently stored at -80°C. Complement-inactivated serum was prepared by incubation of serum for 30 min at 56°C. Human neutrophils were isolated freshly from heparinized blood using the Ficoll-Histopaque gradient method[41] and used on the same day.

Mice

C57BL/6 female mice were purchased from Harlan-Winkelmann (Borchen, Germany) and used in experiments when they were between 8 and 10 weeks of age. They were housed in micro isolator cages and given food and water ad libitum.

Phagocytosis assays

Whole blood phagocytosis. FITC-labeled *S. aureus* KV27 ($1x10^8$/ml) was incubated with freshly isolated human lepirudin blood (50%) and buffer or Efb (0.5 µM) in RPMI-0.05% HSA for 25 min at 37°C. The reaction was stopped using FACS lysing solution (BD Biosciences); samples were washed with RPMI-0.05% HSA and analyzed by flow cytometry using a FACSCalibur (BD). Gating of cells occurred on basis of forward and side scatter; for each sample we measured the fluorescence intensity of 10,000 gated neutrophils. Phagocytosis was expressed as the percentage of neutrophils that became fluorescent.

Phagocytosis with purified neutrophils and plasma/serum. FITC-labeled bacteria ($5x10^7$/ml) were mixed with human serum or plasma for 2 min at 37°C in the presence or absence of Efb. Freshly isolated neutrophils ($5x10^6$/ml) were added and phagocytosis was allowed for 15 min at 37°C. The reaction was stopped by formaldehyde fixation and analyzed by flow cytometry. Alternatively, phagocytosis mixtures were cytospinned on glass slides and

stained using Giemsa-based Diff-Quick solution. To analyze killing, phagocytosis mixtures were not fixed but incubated for an additional 90 minutes before they were diluted into ice-cold water (pH 11) and incubated for 15 min on ice to enable neutrophil lysis. Viable bacteria were quantified by colony enumeration. For Fg supplementation, 5% serum was supplemented with 50-200 µg/ml human or mouse Fg (kindly provided by Dr. Jay L. Degen; purified from plasma of wild type and Fgγ$^{390–396A}$ mice[21]). To analyze the influence of bacterial supernatants on phagocytosis, FITC-labeled *S. aureus* KV27 (2.5x10^7 cfu) was pre-incubated with human serum for 30 min at 37°C in Veronal Buffered Saline containing Ca^{2+} and Mg^{2+} (VBS^{++}). After washing in VBS^{++}-0.5% BSA, bacteria were incubated with (2-fold) diluted culture supernatants or purified Efb (250 nM) for 1 hour at 37°C. After washing, bacteria were incubated with purified Fg (60 µg/ml, Invitrogen) in RPMI-HSA for 1 hour at 37°C and subsequently, neutrophils were added (7.5x10^5 cells) and phagocytosis was allowed for 30 min at 37°C.

In vivo phagocytosis. *S. aureus* strain SH1000[42] was grown to mid-log phase, heat-inactivated for 60 min at 90°C, and fluorescently labeled with carboxyfluorescein (Molecular Probes, Göttingen, Germany). To induce infiltration of neutrophils within the peritoneal cavity, mice were treated i.p. with 1 mg of carrageenan (Type IV1, Sigma) 4 and 2 days prior to bacterial challenge. Subsequently, mice were injected i.p. with 200 µl of a solution containing 10^8 heat-inactivated carboxyfluorescein-labeled *S. aureus* SH1000 and Efb (1 µM). To compare WT and ΔEfb strains, mice were directly inoculated in the peritoneal cavity with 300 µl of GFP-expressing WT or ΔEfb *S. aureus* cultures grown to a late exponential phase. Mice were sacrificed 1 h thereafter, and their peritoneum was lavaged with sterile PBS. Lavage samples were centrifuged and pelleted cells were incubated with purified anti-CD32 antibodies to block the FcR, followed by PE-conjugated anti-mouse Gr-1 antibodies. Cells were washed and quenched with trypan blue (2 mg/ml). Samples were immediately subjected to flow-cytometric analysis using a FACScan (Becton Dickinson, San Jose, California). Neutrophils were gated according to their expression of Gr-1 antigen (FL2). Phagocytosis was expressed as the percentage of neutrophils that became fluorescent.

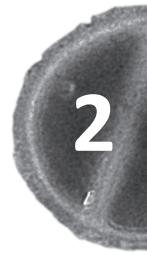
Alternative pathway hemolysis assay

Human serum (5%) was incubated with buffer or Efb proteins (1 µM) in HEPES-MgEGTA (20 mM HEPES, 5mM $MgCl_2$, 10 mM EGTA) for 15 min at RT. Rabbit erythrocytes (Biotrading Benelux B.V.) were added and incubated for 60 min at 37°C. Mixtures were centrifuged and hemolysis was determined by measuring the absorbance of supernatants at 405 nm.

Immunoblotting

To analyze C3b deposition on the bacterial surface, *S. aureus* strain Wood46 (3x10^8/ml) was incubated with 5% human plasma in the presence of Efb (0.5 µM), EDTA (5 mM) or buffer ($HEPES^{++}$; 20 nM HEPES, 5 mM $CaCl_2$, 2.5 mM $MgCl_2$, pH 7.4) for 30 min at 37°C shaking at 1100 rpm. Bacteria were washed twice with PBS-0.1% BSA and boiled in Laemmli sample buffer containing Dithiothreitol. Samples were subjected to SDS-PAGE and subsequently transferred to a nitrocellulose membrane (Millipore). C3b was detected using a peroxidase-labeled polyclonal anti-human C3 antibody (Protos Immunoresearch, Burlingame, USA)

and developed using Enhanced Chemiluminescence (ECL, GE). To quantify Efb in bacterial supernatants, His-Efb and supernatants were run together on an SDS-PAGE gel. After transfer, blots were developed using a polyclonal sheep anti-Efb antibody (kindly provided by Prof JI Flock), peroxidase-labeled donkey anti-sheep antibodies (Fluka Analytical) and ECL. Bands were quantified using ImageJ software and linear regression analysis was performed using GraphPad software.



Flow cytometry assays with *S. aureus*

S. aureus strain Wood46 (3x10^8/ml) was pre-incubated with human serum for 30 min at 37°C in VBS^{++} buffer, washed with VBS^{++}-0.5% BSA and incubated with Efb (0.5 µM) or 2-fold diluted culture supernatants for 1 hour at 37°C shaking. After another washing step, bacteria were incubated with Alexa-488 conjugated Fg (60 µg/ml, Invitrogen) for 1 hour at 37°C shaking. Washed bacteria were analyzed by flow cytometry using a FACSCalibur (BD). Bacteria were gated on the basis of forward and side scatter properties and fluorescence of 10,000 bacteria was analyzed. Alternatively, we incubated pre-opsonized bacteria with Efb (0.5 µM) and/or unlabeled Fg (200 µg/ml) for 1 hour at 37°C shaking. Washed bacteria were incubated with soluble rCR1 (10 µg/ml, kindly provided by Prof. Atkinson, Washington University, St. Louis, MO), FITC-labeled $F(ab')_2$ anti-human C3 antibody (Protos Immunoresearch) or anti-human IgG antibody for 30 min at 37°C. CR1 was detected using PE-labeled anti-CD35 antibodies (BD Pharmingen); the IgG antibody was detected using goat-anti-mouse PE antibodies (BD Pharmingen). Capsule expression on strain Reynolds was analyzed by incubating bacteria with polyclonal anti-CP5 rabbit serum and Phycoerythrin (PE)-conjugated goat anti-rabbit antibody.

Confocal microscopy

Samples were transferred to glass slides and air-dried. Membrane dye FM 5-95 (Invitrogen) was added and slides were covered with a coverslip. Confocal images were obtained using a Leica TCS SP5 inverted microscope equipped with a HCX PL APO 406/0.85 objective (Leica Microsystems, The Netherlands).

Transmission Electron Microscopy

S. aureus strain Wood 46 (3x10^8) was incubated with human plasma (10%) in the presence or absence of Efb (0.5 µM) in $HEPES^{++}$ for 30 min at 37°C, washed once with PBS-1% BSA and adsorbed to 100 mesh hexagonal Formvar-carbon coated copper grids (Stork-Veco, Zoetermeer, NL). Samples were contrasted with 0.4% uranyl acetate (pH 4.0) and 1.8% methylcellulose[43] and analyzed in a JEOL 1010 transmission electron microscope (JEOL Europe, Nieuw Vennep, the Netherlands) at 80 kV.

Statistics

Statistical analyses were performed using GraphPad Prism 4.0 package and the differences between groups were analyzed for significance using the two-tailed Student's *t*-test.

Accession number

The accession number for Efb in *S. aureus* Newman is YP_001332103, locus NWMN_1069.

Acknowledgments

The authors thank the Cell Microscopy Center, Dept of Cell Biology, UMC Utrecht and Manfred Rohde from the Helmholtz Centre for Infection Research, Braunschweig, Germany for great assistance with electron microscopy. We thank Jay L. Degen (Cincinnati) for providing mouse Fg and Fg^Mac1, Jan-Ingmar Flock (Stockholm) for providing anti-Efb antibodies, John Atkinson (St Louis) for providing recombinant CR1, Alexander Horswill (Iowa) for providing pCM29 and Jean Lee (Boston) for sharing the Reynolds (mutant) strain and anti-capsular antibodies. This work was funded by the ALTANT project of the Dutch government.

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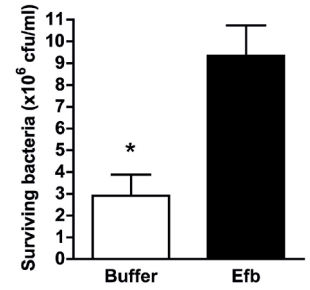
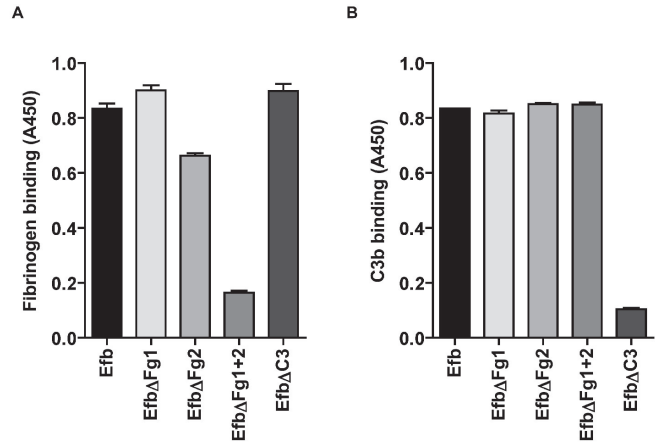
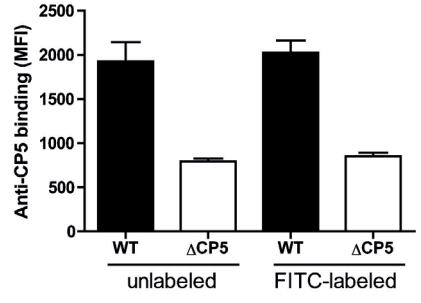
Supplemental information



Supplemental figure 1. Purified Efb inhibits killing of *S. aureus* in human plasma. Killing of *S. aureus* by purified human neutrophils in the presence of 5% human plasma and Efb (0.15 μM). Data are mean ± se of two independent experiments. **P*<0.05 for Efb versus buffer (two-tailed Student's *t*-test).



Supplemental figure 2. Fg- and C3b-binding characteristics of Efb mutants. ELISA experiments analyzing binding of Efb (mutants) to Fg (A) or human C3b (B). Microtiter wells, coated with 0.25 μg human Fg (A) or C3b (B), were incubated with Efb or Efb mutants (both at 6 nM). Efb binding was detected using peroxidase-conjugated rabbit anti-GST antibodies (Abcam). Data are mean ± se of two independent experiments.



Supplemental figure 3. Capsule expression. (FITC-labeled) *S. aureus* strain Reynolds and its isogenic CP5-deficient mutant were incubated with Rabbit-anti-CP5 antibodies and PE-labeled goat-anti-rabbit antibodies. Antibody binding was quantified using flow cytometry. Data are mean ± se of two independent experiments.

Chapter 3

The S. aureus polysaccharide capsule and Efb-dependent fibrinogen shield act in concert to protect against phagocytosis

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Abstract

Staphylococcus aureus has developed many mechanisms to escape from human immune responses. In order to resist phagocytic clearance, *S. aureus* expresses a polysaccharide capsule, which effectively masks the bacterial surface and surface-associated proteins, such as opsonins, from recognition by phagocytic cells. Additionally, secretion of the Extracellular fibrinogen binding protein (Efb) potently blocks phagocytic uptake of the pathogen. Efb creates a fibrinogen shield surrounding the bacteria by simultaneously binding complement C3b and fibrinogen at the bacterial surface. By means of neutrophil phagocytosis assays with fluorescently labeled encapsulated serotype 5 (CP5) and serotype 8 (CP8) strains we now compare the immune-modulating function of these shielding mechanisms. Our data indicate that, in highly encapsulated *S. aureus* strains, the polysaccharide capsule is able to prevent phagocytic uptake at plasma concentrations <10%, but loses its protective ability at higher concentrations of plasma. Interestingly, Efb shows a strong inhibitory effect on both capsule-negative as well as encapsulated strains at all tested plasma concentrations. Furthermore our results suggest that both shielding mechanisms can exist simultaneously and collaborate to provide optimal protection against phagocytosis at a broad range of plasma concentrations. Since opsonizing antibodies will be shielded from recognition by either mechanism, incorporating both capsular polysaccharides and Efb in future vaccines could be of great importance.

Introduction

Staphylococcus aureus (*S. aureus*) is a major human pathogen responsible for many community- and hospital-acquired infections. Disease conditions may range from mild wound infections to more severe invasive illnesses like endocarditis and bacteremia[1,2].

The innate immune system is of high significance for the clearance of invading pathogens such as *S. aureus*[3]. Neutrophils, predominant phagocytic cells of the innate immune system, rapidly engulf bacteria via phagocytosis and kill them intracellularly. Neutrophils recognize bacteria via specific receptors that are directed against bacterium-bound opsonins such as antibodies and complement components. The complement system is a complex proteolytic cascade of human plasma proteins that recognize surface-associated antibodies and specific bacterial surface structures[4,5]. Activation of the cascade will result in deposition of several complement proteins at the bacterial surface. Complement component C3b is the major opsonin responsible for phagocytosis of bacteria by neutrophils and other phagocytic cells. Additionally, interaction of the Fc domain of bacterium-bound antibodies (IgG) with Fc receptors on the neutrophil contributes to effective phagocytosis.

In order to resist phagocytic clearance, *S. aureus* has evolved various immuno-modulatory mechanisms that frustrate the process of phagocytosis[6–10]. For instance, *S. aureus* produces several proteins that modulate binding of IgG to the bacterial surface (protein A and Sbi) or inhibit recognition of surface-bound IgG by Fc receptors (FLIPr). Also, *S. aureus* secretes multiple proteins that block activation of complement (e.g., SCIN, Ecb, Efb, Cna, SSL10). Furthermore, *S. aureus* has developed several ways to shield its surface from recognition by the host immune system. The first shielding mechanism is represented by the formation of a capsule, a polysaccharide structure surrounding the bacterial cell wall[11]. The two main serotypes produced by clinical *S. aureus* strains are the serotype consisting of capsular polysaccharide 5 (CP5) and capsular polysaccharide 8 (CP8), accounting for ~75% of all clinical isolates, of which CP8 strains are the most prevalent[12–15]. These capsules comprise trisaccharide repeating units of N-acetyl mannosaminuronic acid, N-acetyl L-fucosamine and N-acetyl D-fucosamine and are identical except for the glycosidic linkages between the sugars and the sites of O-acetylation[16]. The CP5 and CP8 strains form non-mucoid colonies that are indistinguishable from colonies formed by unencapsulated strains. CP5 and 8 were not only found among clinical isolates but are also expressed by commensal strains[13,14]. The expression of CP5 or CP8 has been shown to enhance virulence and survival of *S. aureus in vivo*[17–19]. Next to inhibition of phagocytic uptake, CP5 expression has been described to provide protection against intracellular killing of the bacterium[18]. However, *S. aureus* capsule expression (and therefore capsule size) is highly variable and depends on the presence or absence of certain environmental factors, such as CO_2 [20]. Therefore, capsule density and thus inhibition of phagocytosis are subject to the location of the bacterium in the body.

As a second shielding mechanism against phagocytosis, *S. aureus* secretes a protein that links specific plasma proteins to its surface. This Extracellular fibrinogen binding protein (Efb) is a 16 kDa protein that binds to complement C3b on bacteria and simultaneously attracts fibrinogen to the surface. In doing so, Efb covers bacteria with a thick layer of fibrinogen that potently prevents recognition of surface-associated antibodies and C3b by

phagocytic cells[21].
Currently, it is not well understood why *S. aureus* evolved two separate mechanisms for shielding its surface from phagocytosis. In this study we further analyze the antiphagocytic properties of both the capsule and Efb. Our findings indicate that these two shielding mechanisms can work in concert to enhance the resistance of *S. aureus* against phagocytosis.

Results

Fluorescent labeling and capsule expression of *S. aureus* strains

In order to compare the antiphagocytic effects of the staphylococcal polysaccharide capsule with the Efb shield, we first performed *in vitro* neutrophil phagocytosis assays with different encapsulated *S. aureus* strains that show varying degrees of capsule expression (**Supplemental figure 1**). Since the Reynolds strain expresses a thick CP5 capsule (**Supplemental figure 1** and [17,19]), we initially focused on this strain in our phagocytosis experiments and used its isogenic mutant (Reynolds CP$^-$) as a capsule-negative strain. Both strains were fluorescently labeled by transformation with a pCM29-GFP plasmid that allows for intracellular production of GFP under a constitutive promoter [23]. Strains were grown on Columbia agar supplemented with 2% NaCl (CSA) for 24 hours at 37°C to guarantee that capsule expression was optimal [17]. The fluorescence of the strains was measured by flow cytometry (**figure 1A**), which confirmed that GFP was properly expressed and that both strains were equally fluorescent. Furthermore, we confirmed expression of the polysaccharide capsule after fluorescent labeling by specific staining with a polyclonal antibody directed against CP5 (**figure 1B**). Finally, using transmission electron microscopy, we visualized the polysaccharide capsule of the GFP-labeled Reynolds (CP5) strain (**figure 1C**).

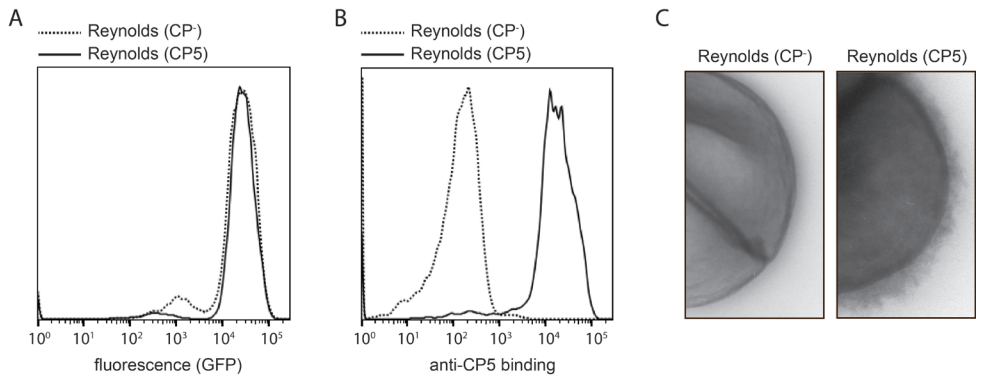

Figure 1. Fluorescent labeling and capsule expression of *S. aureus* strains. **A.** Flow cytometry histogram showing the fluorescence of *S. aureus* strain Reynolds (CP5) and its isogenic capsule-negative mutant (CP$^-$) after transformation with pCM29-GFP plasmid. **B.** Flow cytometry histogram showing binding of rabbit anti-CP5 antibodies to GFP-labeled Reynolds (CP5) and (CP$^-$), detected with Alexa647-conjugated protein A. **C.** Transmission electron microscopy images showing the GFP-labeled Reynolds (CP$^-$) and (CP5) strain. Strains were pretreated with anti-CP5 antibodies to enhance stability and electron density of the capsule. **A-C.** Representative images are shown.

The polysaccharide capsule and Efb together protect against phagocytosis at a broad range of plasma concentrations

After confirming expression levels of both fluorescence and polysaccharide capsule, the strains were analyzed in phagocytosis assays. We incubated the GFP-labeled *S. aureus* Reynolds (CP5) and (CP$^-$) strain with normal human plasma (as a source for antibodies, complement and fibrinogen) and freshly isolated human neutrophils. Phagocytosis of fluorescent bacteria by neutrophils was quantified using flow cytometry. As expected, we

observed that the unencapsulated *S. aureus* strain was efficiently phagocytosed (**figure 2A**). As described previously[17,28], the polysaccharide capsule of the Reynolds (CP5) strain potently blocked neutrophil phagocytosis at low plasma concentrations (**figure 2A**). However, at plasma concentrations ≥10%, we observed little to no difference between the CP5-expressing Reynolds strain and its isogenic capsule-negative mutant. This suggests that CP5 does not protect against phagocytosis at higher plasma concentrations. Since we previously observed that Efb prevents phagocytosis of *S. aureus* in plasma by shielding the bacterial surface with fibrinogen[21], we wondered whether addition of Efb could also affect phagocytosis of encapsulated *S. aureus* strains. First, we found that purified GST-tagged Efb (0.5 μM) significantly blocked phagocytosis of the capsule-negative Reynolds (CP^-) strain at all tested plasma concentrations (**figure 2B**). When the encapsulated strain Reynolds (CP5) was used, Efb also had an inhibitory effect on bacterial uptake at higher plasma concentrations, where the polysaccharide capsule itself is no longer protective (**figure 2C**). As a control, we showed that the GST-tag alone or GST-tagged Efb-N (the N-terminal domain of Efb) did not reduce phagocytic uptake (data not shown). These results suggest that the polysaccharide capsule and the Efb-dependent fibrinogen shield collaborate to fully protect *S. aureus* at an extensive range of plasma concentrations. Altogether, our findings indicate that these two antiphagocytic mechanisms collaborate to fully protect *S. aureus* at an extensive range of plasma concentrations.

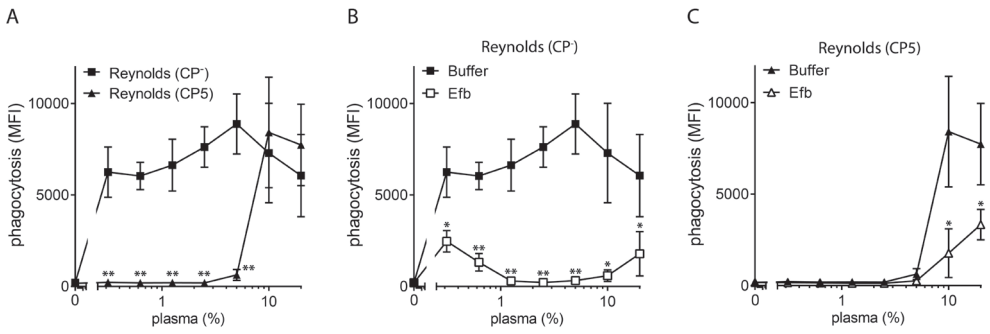

Figure 2. The polysaccharide capsule and Efb together protect against phagocytosis at a broad range of plasma concentrations. Phagocytosis of GFP-labeled Reynolds (CP^-) and (CP5) by purified human neutrophils in the presence of human plasma alone (**A**) or plasma with 0.5 μM Efb (**B-C**), measured by fluorescence (geomean) of the neutrophils. **A.** The polysaccharide capsule of *S. aureus* provides protection against phagocytosis in low plasma concentrations. **B.** Addition of exogenous Efb inhibits phagocytic uptake of unencapsulated strain Reynolds (CP^-) in all tested plasma concentrations. **C.** Inhibition of phagocytosis of encapsulated strain Reynolds (CP5) is enhanced by addition of Efb. Graphs represent mean ± sd of three separate experiments. **P*<0.05, ***P*<0.005 for Reynolds (CP5) versus Reynolds (CP^-) or Efb versus Buffer (two-tailed Student's *t*-test).

The Efb-dependent fibrinogen shield provides protection against phagocytosis on various encapsulated and capsule-negative *S. aureus* strains

Since we used the highly encapsulated Reynolds strain in these experiments, we wondered whether the presence of different plasma concentrations also influences phagocytosis inhibition by other capsule-expressing *S. aureus* strains. We therefore performed

phagocytosis assays in 1, 3 and 10% plasma using either unencapsulated *S. aureus* strains (USA300 and Wood 46), CP5-expressing strains (Col and USA100) and CP8-expressing strains (Sanger 252 and Reynolds CP8 , an isogenic mutant of strain Reynolds in which the *cap5* region was substituted with *cap8*). All strains were labeled with GFP and grown on CSA to ensure optimal capsule expression (**Supplemental figure 1**). Since the absolute fluorescence levels varied between strains, we expressed phagocytic uptake of each strain (**Figure 3A**) as a relative value compared to the MFI at 10% plasma, at which phagocytosis had reached its maximum. Although this prohibits direct comparison between strains, this still allows us to analyze the effect of different plasma concentrations on phagocytosis efficiencies of each strain. Similar to the capsule-negative Reynolds strain, none of the other capsule-negative strains (USA300, 8325-4, Wood 46) showed a significant difference in phagocytic uptake at lower concentrations of plasma, compared to 10% plasma (**figure 3A**). Notably, the other CP5- and CP8-expressing strains (COL, USA100 Sanger 252) did not show a substantial decrease in phagocytosis at the lower plasma concentrations. Only the isogenic Reynolds CP8 mutant showed a reduction in phagocytosis at 1% plasma concentration. These experiments suggest that the antiphagocytic effect of the *S. aureus* capsule depends both on the expression level of the capsule and the plasma concentration. Next, we tested the antiphagocytic effect of Efb on the other GFP-labeled CP5- and CP8-expressing strains. We observed that addition of Efb significantly reduced phagocytic uptake of all tested CP^-, CP5 and CP8 strains at both 1% as well as 10% plasma (**figure 3B**). Since phagocytosis of strain Reynolds (CP5) and (CP8) was already considerably reduced at 1% plasma, an additional significant decrease in phagocytic uptake in the presence of Efb was not measured. This shows that the Efb-dependent fibrinogen shield can be created and function properly on capsule-negative as well as capsule-expressing *S. aureus* strains.

Interplay between Efb and capsule on other isogenic mutants of CP5 and CP8

The phagocytosis experiment in Figure 3B suggests that capsule-mediated inhibition in our assay system is only detectable for strain Reynolds, but not for other *S. aureus* strains. However, the exact contribution of the capsule in this experiment could not be studied due to the lack of isogenic capsule-negative mutants. Therefore, we decided to include three different capsule-expressing *S. aureus* strains (Newman (CP5), Becker (CP8) and MN8 (CP8)) in which the *cap5* and *cap8* loci are deleted[19]. These strains were fluorescently labeled and capsule expression was determined using specific CP5 and CP8 antibodies (**figure 4A**). When phagocytosis was analyzed, results with FITC-labeled strain Reynolds (CP5) and (CP^-) were comparable to previous assays; at 1% plasma the encapsulated strain showed a decrease in phagocytic uptake compared to the capsule-negative strain but at 10% plasma this inhibitory effect was not present (**figure 4B**). Strikingly, the polysaccharide capsule of strain Newman, showing 74% CP5 expression compared to Reynolds, was not able to block phagocytosis at 1 and 10% plasma (**figure 4C**). Also, the capsules of strain Becker and MN8 showed no significant inhibition of phagocytosis (**figure 4D, E**). As anticipated, addition of Efb blocked the phagocytic uptake of all tested strains, regardless of their capsule expression (**figure 4B-E**). Again, this shows that the Efb-dependent fibrinogen shield can be created and function properly on capsule-negative as well as capsule-expressing *S. aureus* strains.

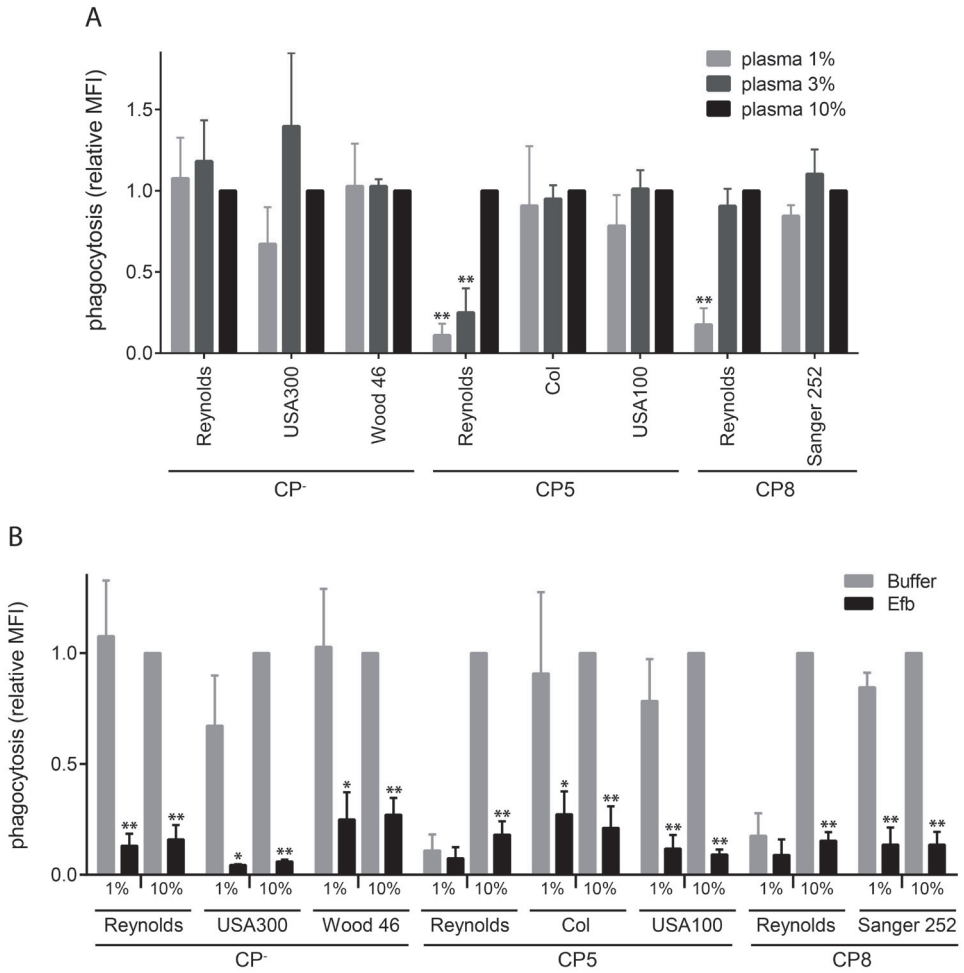

Figure 3. The Efb-dependent fibrinogen shield provides protection against phagocytosis on various encapsulated and capsule-negative *S. aureus* strains. A. Phagocytosis of different GFP-labeled CP$^-$-, CP5- and CP8-expressing *S. aureus* strains in the presence of 1, 3 or 10% human plasma. Phagocytosis is displayed as the relative fluorescence compared to the 10% plasma condition of each strain. Graph represents mean ± sd of three separate experiments. **P*<0.05, ***P*<0.005 for plasma 1% or 3% versus plasma 10% of the same strain (two-tailed Student's *t*-test). **B**. Addition of Efb inhibits phagocytic uptake of different CP5 and CP8 encapsulated *S. aureus* strains at both 1% and 10% human plasma. This was displayed by the relative fluorescence (geomean; compared to the Buffer condition at 10% plasma of the same strain) of the neutrophils. Graph represent mean ± sd of three separate experiments. **P*<0.05, ***P*<0.005 for Efb versus Buffer of the same strain (two-tailed Student's *t*-test).

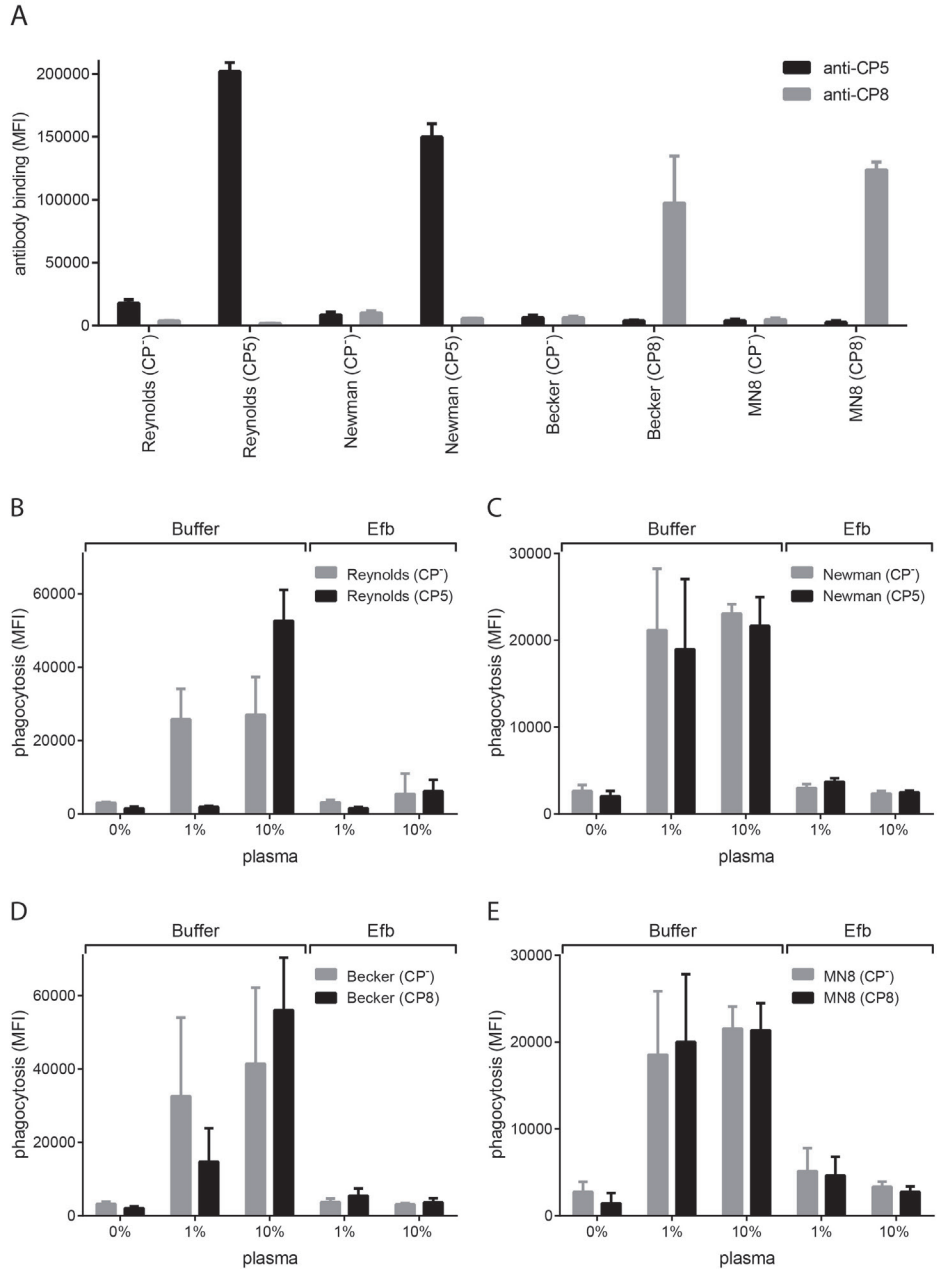

Figure 4. Interplay between Efb and capsule on other isogenic mutants of CP5 and CP8. A. Binding of rabbit CP5 and CP8 antibodies to different FITC-labeled CP5- and CP8-expressing *S. aureus* strains. **B-E**. Phagocytosis of different FITC-labeled CP5- and CP8-expressing *S. aureus* strains by purified neutrophils in the presence of 1 or 10% human plasma and 0.5 µM Efb. **B**. Phagocytosis of strain Reynolds (CP5) and Reynolds (CP$^-$) **C**. Phagocytosis of CP5-expressing strain Newman and its isogenic mutant Newman (CP$^-$) (Buffer vs Efb: *P*<0.05 at 1% and 10% plasma). **D**. Phagocytosis of CP8-expressing strain Becker and its isogenic mutant Becker (CP$^-$) (Buffer vs Efb: n.s. at 1% plasma, *P*<0.05 at 10% plasma). **E**. Phagocytosis of CP8-expressing strain MN8 and its isogenic mutant MN8 (CP$^-$) (Buffer vs Efb: *P*<0.05 at 1% and 10% plasma). Graphs represent mean ± sd of three separate experiments.

At 10% plasma, the inhibitory effect of Efb was statiscally significant for all strains, however at 1% plasma only for strain Newman and MN8.

Efb attracts fibrinogen to the surface of both CP$^-$ and CP5 strains

To confirm that Efb can indeed create a fibrinogen shield around the surface of encapsulated strains, we visualized this shield by confocal microscopy. We pre-opsonized mCherry-labeled Reynolds (CP$^-$) and (CP5) with 3% plasma and incubated them with Alexa488-labeled fibrinogen in the presence of Efb (0.5 µM). As a control, we included two Efb mutant proteins that cannot form this shield due to the lack of fibrinogen (EfbΔFg) or C3b (EfbΔC3) binding motifs. First, we observed that bacteria incubated without the addition of Efb did not show binding of fibrinogen to the bacterial surface, which was to be expected since washed bacteria were used and therefore no endogenously produced Efb was present (**figure 5A**). In the presence of Efb, both the CP$^-$ and CP5 strain were completely surrounded by a layer of fluorescent fibrinogen. This fibrinogen layer was not present when bacteria were incubated with the Efb mutant proteins. These results were confirmed by flow cytometry analyses of the samples used for confocal microscopy, showing a considerable increase of fibrinogen binding in the presence of full-length Efb (**figure 5B**). Remarkably, no significant difference was observed between the CP$^-$ and CP5 strain incubated with full-length Efb in both confocal microscopy as well as flow cytometry. This suggests that formation of the Efb-dependent fibrinogen shield is equally efficient on encapsulated and capsule-negative strains.

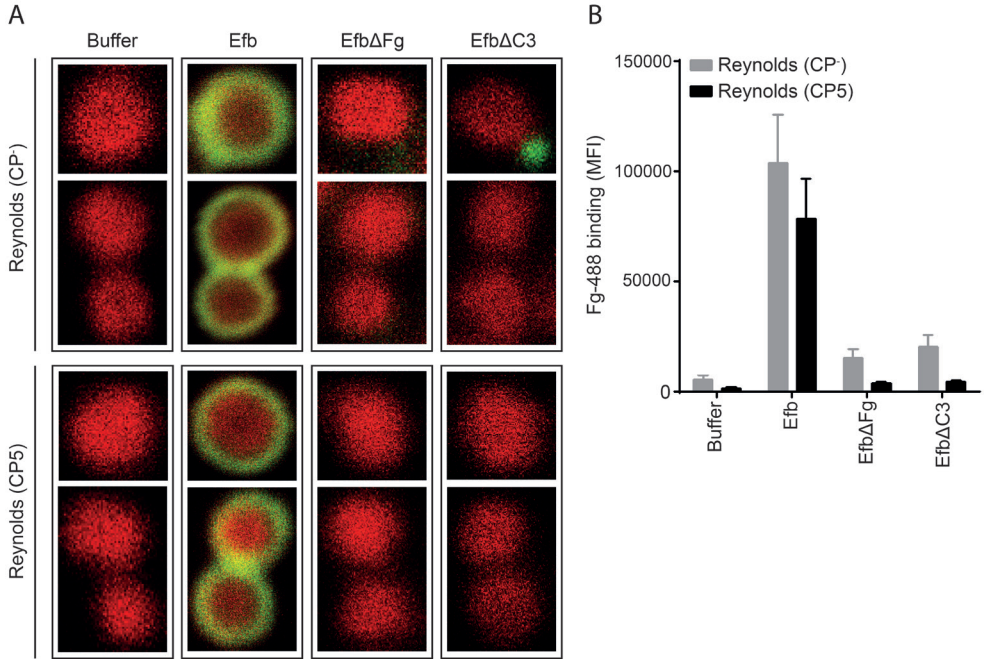

Figure 5. Efb attracts fibrinogen to the surface of both CP$^-$ and CP5 strains. A. Confocal images of the binding of Alexa488-labeled fibrinogen to mCherry-labeled Reynolds (CP$^-$) and (CP5) strains, pre-opsonized with human serum (3%), in the presence of Efb variants (0.5 µM). Representative images are shown. **B.** Flow cytometry analyses of samples shown in A. Graph represents mean ± sd of three separate experiments. ***P*<0.005 for Efb versus Buffer, EfbΔFg or EfbΔC3 (two-tailed Student's *t*-test).

Discussion

S. aureus has evolved many ways to evade and manipulate immune responses in order to survive inside the human host [6]. Since phagocytic uptake of *S. aureus* by neutrophils is crucial for the clearance of the pathogen, suppressing this process will be of great importance to its persistence in the body. The polysaccharide capsule expressed by *S. aureus* has been shown to potently block killing by human neutrophils by covering C3b attached to the bacterial surface [17,19]. In this study, we observe that the capsule of strain Reynolds (CP5) and (CP8) can efficiently block phagocytosis at low concentrations of plasma but that it loses its protective capacity at higher plasma concentrations. Furthermore, we have previously shown that Efb forms a shield of fibrinogen and thereby protects bacteria from phagocytosis[21]. Now, we demonstrate that the Efb-dependent fibrinogen shield can also effectively be formed on several encapsulated strains at a broad range of plasma concentrations. This shows that these two mechanisms of shielding can collaborate to ensure optimal protection against phagocytosis.

The results presented here suggest that the polysaccharide capsule of strain Reynolds has strong antiphagocytic properties at lower plasma concentrations. Whereas the polysaccharide capsule has previously been shown to prevent recognition of staphylococcal surface-associated proteins by neutrophil receptors, it does not completely block the binding of specific antibodies nor the deposition of complement components at the bacterial surface[19,29,30]. Complement activation was triggered by encapsulated *S. aureus*, which resulted in rapid deposition of C3b. However, purified capsular polysaccharides are not immunogenic and did not trigger complement activation[19,31]. Hence, deposition of C3b on encapsulated strains does occur but was described to be located underneath the polysaccharide capsule on the bacterial cell wall and is thereby shielded from its surroundings[19]. Because C3b is able to deposit on top of other C3b molecules[32], it is possible that in high concentrations of plasma, C3b molecules can accumulate and eventually be displayed above the capsule, no longer shielded from complement receptors. This may explain the lack of phagocytic resistance by the polysaccharide capsule at high plasma concentrations in this study. An alternative explanation would be that antibodies against the polysaccharide capsule are present in normal human plasma, although concentrations have been shown to be too low to mediate phagocytic uptake[17,33]. However, we previously showed that specific capsular antibodies can potently neutralize the antiphagocytic effect of the polysaccharide capsule, since these antibodies enhance phagocytosis of encapsulated strains at low plasma concentrations[21]. This could indicate that at high plasma concentration, levels of antibodies directed against capsular polysaccharides are sufficient to efficiently activate complement, and thus C3b deposition, on top of the capsule leading to phagocytic uptake or to directly mediate phagocytosis through recognition by Fc receptors. Nevertheless, not all capsule-expressing strains tested in this study showed similar shielding capacities. Inhibition was most potent for the Reynolds (CP5) strain that is known for its thick capsule. CP8 and CP5 strains have been described to differ in their virulence, explained by the suggestion that CP5 strains commonly express more capsular polysaccharides than CP8 strains[19]. However, our *in vitro* data will not predict capsule expression inside the body and it is therefore also possible

that these strains do produce a dense capsule *in vivo,* which is effective against phagocytic uptake. Interestingly, it was reported that the highly virulent USA300 isolates, prevalent in North-America, lack the expression of capsular polysaccharide[34]. Also here, we observe that a USA300 isolate does not show impaired phagocytosis. Possibly, these strains use other mechanisms to circumvent phagocytic killing. For instance, expression of Efb and Clumping factor A (ClfA) have been shown to be upregulated in USA300 strain LAC and therefore the fibrinogen binding capacity of this strain is high [35]. Also, higher production of molecules that directly lyse neutrophils, such as Panton Valentine Leukocidin (PVL) and Phenol Soluble Modulin (PSM) could compensate for the lack of capsule expression by USA300 isolates[35,36]. Furthermore, our results indicate that Efb most potently prevents phagocytosis of the capsule-negative strain at plasma concentrations between 1% and 10%. For Efb to completely cover *S. aureus* with a shield of fibrinogen and thus fully block phagocytosis, it not only requires simultaneous binding to both C3b and fibrinogen but also, very importantly, sufficient levels of these two plasma proteins. This explains the reduced efficiency of the Efb-dependent fibrinogen shield at very low plasma concentrations, since the layer generated at these levels of complement and fibrinogen will not be dense enough to completely mask the bacterial surface. We now show that Efb is also able to establish strong inhibition of phagocytosis on highly encapsulated strains. Therefore, binding of Efb seems not to be affected by the presence of capsular polysaccharides, even those of the highly encapsulated Reynolds (CP5). Since the two shielding mechanisms provide protection at both low and high plasma concentrations, this could suggest that *S. aureus* has the ability to shield itself from phagocytic uptake at different locations inside the host, from tissue to bloodstream. Although we do not provide direct evidence that these shielding mechanisms occur during an infection *in vivo*, we believe that the concentrations of Efb used are relevant. Previously, we quantified the secretion of Efb in *S. aureus* (strain Newman) culture supernatants and found production levels of ~1 μM[21]. Although strain Newman has higher expression levels of Efb than most *S. aureus* strains due to a point mutation in the SaeR/S regulatory system[37], these levels are still more than 10 times higher than the calculated IC_{50} (0.08 μM) needed for phagocytosis inhibition. Additionally, we showed that endogenously produced Efb mediates complex formation on the bacterial surface, since WT supernatants can attract fibrinogen to the bacterial surface whereas Efb-deficient supernatants do not introduce shield formation[21]. Furthermore, studies of the effect of Efb on virulence of *S. aureus in vivo* show that the protein is expressed at levels high enough to be effective[21,38,39]. Altogether, data presented in this paper indicate that the balance between bacteria, plasma components and infiltrating immune cells can influence the antiphagocytic properties of pathogenic *S. aureus*. Although it is generally believed that whole blood mimics a relevant physiological condition for *S. aureus* infections, we know that most *S. aureus* infections occur at localized sites of the body where bacteria encounter different concentrations of plasma and immune cells than in human whole blood. Furthermore, during an infection, the inflammatory response will alter the plasma-to-immune cell ratio because of rapid influx of immune cells. For this reason, the bacterium may have evolved additional mechanisms to subvert phagocytosis at different concentrations of plasma and neutrophils. This allows the bacterium to subvert immune clearance from different sites of the body and during different stages of an infection.

S. aureus is rapidly becoming more resistant to antibiotics[40] and new therapy strategies are

being explored[41]. Despite interesting developments in preclinical studies[42–44], an effective vaccine against *S. aureus* is still not available. Although clinical studies in humans indicate that opsonic antibodies are successfully produced upon vaccination with different *S. aureus* antigens (including capsular polysaccharides)[45,46], such antibodies fail to protect humans against *S. aureus* infections[47,48]. Possibly, the shielding mechanisms described in this study complicate the effector mechanism of opsonic antibodies. Therefore, the inclusion of both capsular antigens and Efb could be important in the development of a protective *S. aureus* vaccine.

Materials & Methods

Bacterial strains and fluorescent labeling

In this study we used various wild-type *S. aureus* strains expressing different capsular polysaccharides: wild-type CP5-expressing strains include strain Reynolds (Jean Lee, Boston), COL (Andreas Peschel, Tuebingen), USA100 (Jean Lee, Boston) and Newman (Jean Lee, Boston); wild-type CP8-expressing strains include Sanger 252 (Tim Foster, Dublin), Becker and MN8 (Jean Lee, Boston). Capsule-negative strains included are USA300 (Dr. Frank Deleo, NIAID, Hamilton), 8325-4 (Tim Foster, Dublin) and Wood 46 (ATCC-10832). Isogenic capsule-negative mutants of strain Reynolds, Newman, Becker and MN8 were created by deletion of the *cap5 or cap8* as described[19,22]. CP8-expressing strain Reynolds was generated by substitution of the *cap5* region with the *cap8* region[19]. Capsular serotypes were verified by flow cytometry analyses using specific CP5 and CP8 antisera (see below). Strains were fluorescently labeled by transformation with the pCM29 plasmid, constitutively expressing either GFP or mCherry under regulation of the sarA promoter as previously described[23,24]. Alternatively, strains were fluorescently labeled with fluorescein isothiocyanate (FITC, Sigma). To this end, bacteria were grown on Columbia agar (Oxoid) supplemented with 2% NaCl (CSA) for 24 hours at 37°C, suspended, washed and resuspended in PBS. FITC (0.5 mg/ml in DMSO) was added and incubated for 30 min on ice. Bacteria were washed twice and resuspended in RPMI containing 0.05% human serum albumin (RPMI-HSA). All strains were grown on CSA for 24 hours at 37°C to guarantee optimal capsule expression[17,22] and stored at -20°C in RPMI-HSA before use.

Protein purification

Recombinant Efb proteins were generated in *E. coli* as described previously[25]. Briefly, the *efb* gene from *S. aureus* strain Newman (Mal Horsburgh, Liverpool) (without the signal peptide) was amplified by PCR and ligated into the pGEX-5x-1 vector (GE healthcare) for N-terminal fusion with glutathione S-transferase (GST). Mutations of the fibrinogen and C3 binding domains were introduced in pGEX plasmids containing full-length GST-Efb as described previously[21,25]. The mutant EfbΔFg lacks both fibrinogen binding domains and was previously described as EfbΔFg1+2 [21]. EfbΔC3 has been altered in the C3d binding site and therefore lacks C3-binding ability. Recombinant proteins were expressed and purified according to the manufacturer's manual.

Purification of human plasma and neutrophils

In order to prepare plasma, blood was collected in 3 ml blood tubes (Roche) containing recombinant hirudin (15 µg/ml) from four healthy volunteers. After centrifugation for 10 min at 2080g plasma was collected, pooled and stored at -80°C. For the isolation of human neutrophils, blood from a healthy donor was collected in heparin vacutainers (BD) and cells were isolated using the Ficoll-Histopaque gradient method[26].

Capsule visualization with transmission electron microscopy

GFP-labeled *S. aureus* strains Reynolds (CP5) and its isogenic CP-negative mutant ($5*10^7$/ml) were incubated with rabbit CP5 antiserum ([19], 1:100) in PBS-0.5% bovine serum albumin (BSA) for 45 min at 4°C and washed twice with PBS-0.5% BSA. Subsequently, bacteria were adsorbed to 100 mesh hexagonal Formvar-carbon coated copper grids (Stork-Veco, Zoetermeer, NL). Samples were contrasted with 0.4% uranyl acetate (pH 4.0) and 1.8% methylcellulose[27] and analyzed in a Tecnai 12 transmission electron microscope (FEI) at 80 kV.

Capsule quantification by flow cytometry

GFP- or FITC-labeled *S. aureus* strains ($5*10^7$/ml) were incubated with rabbit CP5 and CP8 antiserum ([19], 1:100) in PBS-0.5% BSA for 45 min at 4°C and washed twice with PBS-0.5% BSA. Bacteria were incubated with Alexa647-conjugated Protein A (1:1000, Molecular Probes) and, after another washing step, fixed with formaldehyde (1%) before flow cytometry measurement with a FACS Verse (BD).

Phagocytosis assays

All phagocytosis assays were performed in Falcon® tubes (Corning). Freshly isolated human neutrophils ($5*10^6$/ml) were stained with Vybrant® DiD cell-labeling solution (1:1000, Molecular Probes), washed 3x with and resuspended in RPMI-HSA before use. GFP- or FITC-labeled *S. aureus* ($5*10^7$/ml) were pre-incubated with human plasma in the presence or absence of Efb (0.5 µM) for 2 min at 37°C. DiD-stained neutrophils ($5*10^6$/ml) were added and phagocytosis was allowed for 15 min at 37°C, shaking (600 rpm). Cold formaldehyde (1%) in RPMI-HSA was added to stop the reaction and samples were analyzed by flow cytometry measurement of the fluorescence of the neutrophils.

Confocal microscopy

S. aureus strains Reynolds (CP5 and CP^-) (mCherry-labeled, $1*10^8$/ml) were pre-incubated with human plasma (3%) for 30 min at 37°C in Veronal buffer containing 5 mM $CaCl_2$ and 2.5 mM $MgCl_2$ (VBS^{++}) to deposit C3b on the bacterial surface. After a washing step with VBS++-0.5% BSA, bacteria were incubated with Efb or Efb mutants (0.5 µM) for 1 hour at 37°C, shaking (600 rpm). Following another washing step, a 1 hour incubation with Alexa-488 conjugated fibrinogen (60 µg/ml, Invitrogen) at 37°C shaking was performed, after which bacteria were fixed with formaldehyde (1%). For visualization by confocal microscopy, samples were transferred onto poly-L-lysine coated cover slips (0.45 µm; 12 mm diameter; Becton Dickinson) or as a control, samples were analyzed by flow cytometry. Confocal images were acquired using a Leica TCS SP5 inverted microscope equipped with HCX PL APO CS 63×/1.40–0.60 OIL objective (Leica Microsystems, The Netherlands).

Acknowledgements

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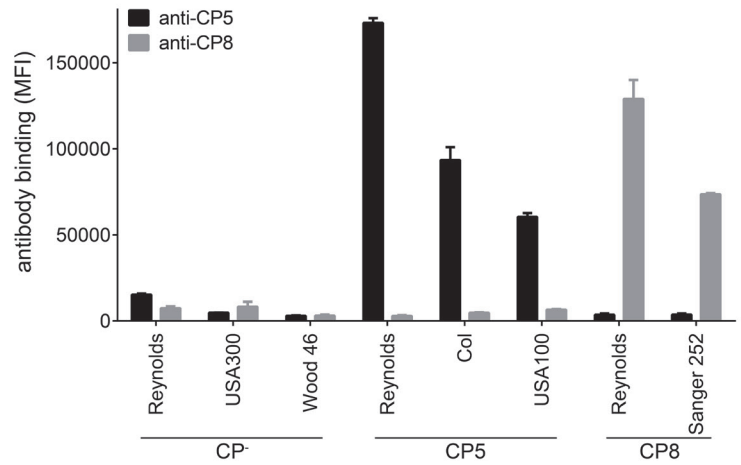
Supplemental information



Supplemental figure 1. Capsule expression of different FITC-labeled encapsulated S. aureus strains. Capsule expression was quantified by incubation with polyclonal anti-CP5 or CP8 antibodies and Alexa647-conjugated protein A, followed by flow cytometry.

Chapter 4

Complement activation by human antibodies against *Staphylococcus aureus* is governed by IgG hexamer formation at the bacterial cell surface

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Manuscript in preparation & Patent pending

Abstract

Antibody therapy represents an alternative approach to the treatment of infections with antibiotic-resistant bacteria such as *Staphylococcus aureus*. It has been suggested that optimal complement activation by IgG molecules requires antibody molecules to arrange into hexameric structures upon target binding on the surface of tumor cells. IgG hexamerization on the cell surface is mediated through non-covalent Fc-Fc interactions, which can be manipulated by specific point mutations in the Fc domain to enhance complement activation. Here, we investigate whether antibody clustering also occurs on the surface of *S. aureus* and whether this process is important for the activation of the complement system on bacteria. Using a peptide that disrupts IgG hexamerization by inhibiting intermolecular Fc-Fc interactions, we demonstrate that deposition of complement components on *S. aureus* in human immune sera and subsequent bacterial uptake by neutrophils is strongly dependent on IgG Fc-Fc contacts. Furthermore, we show that monoclonal antibodies against *S. aureus* wall teichoic acid or capsular polysaccharides, harboring a mutation that enhances hexamer formation, induce increased complement deposition and phagocytosis of bacteria. Antibodies with increased potency to activate human immune defenses provide promising treatment options for infections with *S. aureus* and other multi-drug resistant bacteria.

Introduction

Staphylococcus aureus (*S. aureus*) is a prominent human pathogen causing a wide spectrum of diseases, ranging from mild skin and soft tissue infections to life-threatening conditions like pneumonia and sepsis. Treatment of *S. aureus* infections in hospitalized patients is complicated by the rise of antibiotic-resistant bacteria (MRSA, VRSA)[1]. Monoclonal antibody therapy is considered an attractive strategy to treat or prevent infections with antibiotic-resistant bacteria. In theory, therapeutic antibodies could facilitate treatment of bacterial infection via various modes of action, for instance to a) neutralize bacterial virulence factors[2,3], b) specifically kill intracellular bacteria via antibody-antibiotic drug conjugates[4], or c) to enhance natural eradication of bacteria by human immune defenses. In humans, host clearance of *S. aureus* critically depends on proper engulfment and intracellular killing by neutrophils, as evidenced by recurrent *S. aureus* infections in patients with neutrophil defects[5,6]. In order to effectively engulf *S. aureus*, neutrophils require the help of the complement system, an extensive network of plasma proteins. Upon contact with bacteria, the complement proteins organize into a cascade of proteolytic events that eventually results in massive labeling of bacterial surfaces with complement proteins C3b and iC3b. These 'opsonic' C3b/iC3b molecules potently enhance phagocytosis efficiency via interaction with complement receptors on phagocytic cells. The classical complement pathway (CP) is an important route to trigger the complement cascade on bacteria[7–9]. This pathway is initiated by C1q, a hexamer of globular heads that bind bacterium-bound antibodies (IgG or IgM). Upon binding, C1q activates its associated enzyme C1s to cleave components C4 and C2 to form a C3 convertase enzyme (C4b2a). This C3 convertase, attached to the surface via C4b, rapidly catalyzes the covalent deposition of C3b molecules onto the bacterial surface. C1 binding and subsequent activation of the classical pathway of complement have been shown to be of high importance for the clearance of antibody-coated bacteria and C1 deficiencies will therefore lead to susceptibility for infection[10].

Recently, it became clear that classical pathway activation requires specific arrangement of IgG molecules into hexameric structures[11] (**figure 1A**). While it was previously anticipated that C1q would bind one or two IgG molecules, Diebolder *et al.* proposed that complement activation on tumor cells is most efficient when IgG molecules cluster via non-covalent Fc-Fc interactions. It was suggested that an IgG hexamer is the optimal form for complement activation since the six antibody-binding headpieces of C1q simultaneously bind to hexameric IgG (**figure 1B**). Specific mutations in the Fc domain were identified that enhanced complement-dependent lysis of tumor cells by monoclonal antibodies[11] (**figure 1C**). Currently, it is not known whether antibody multimerization is also relevant for the immune response against infections. Here, we report that Fc-Fc contacts between naturally occurring antibodies against *S. aureus* are criticial to induce classical pathway activation and phagocytosis by neutrophils. Furthermore, we engineer monoclonal antibodies against several *S. aureus* surface epitopes with enhanced capacity to stimulate human immune defenses.

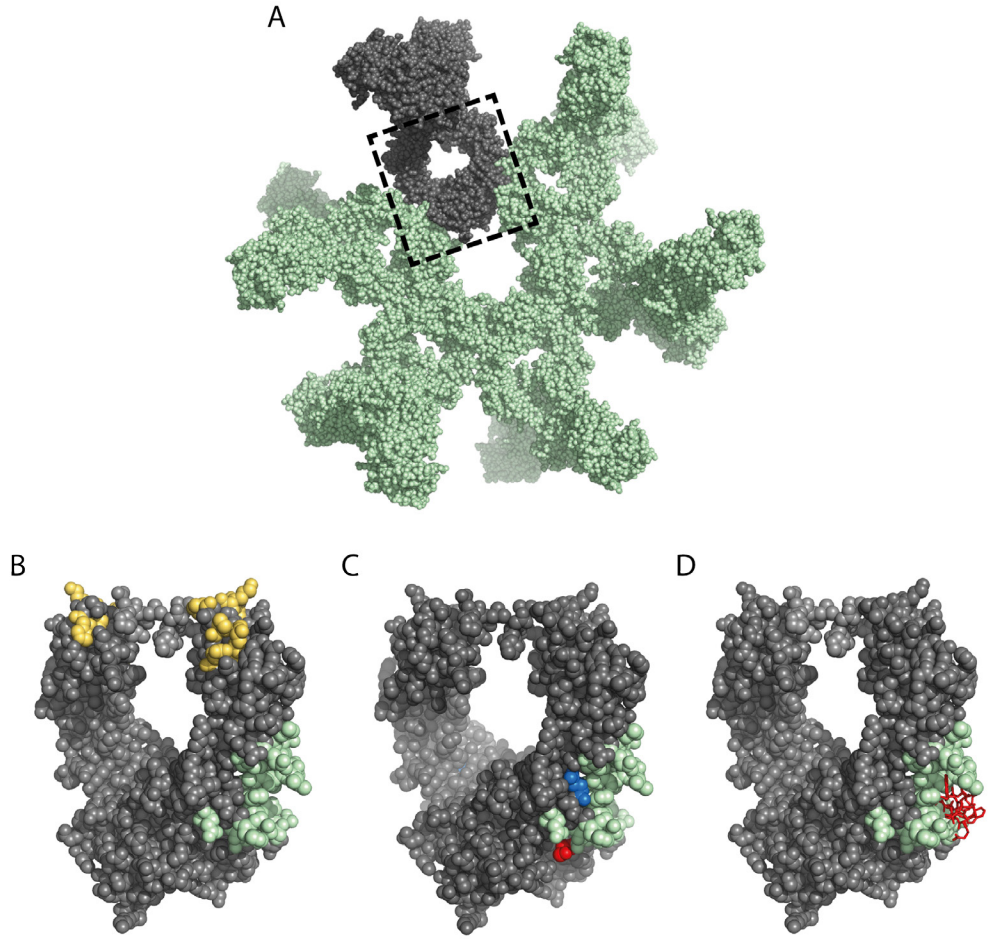


Figure 1. A. IgG hexamer crystal packing of IgG1-b12 (pdb 1HZH[33]). A single IgG is shown in grey. The dashed enclosure indicates the Fc domain. **B-D.** Surface map depicting the Fc domain. Residues involved in Fc-Fc interactions are shown in green[11]. **B.** Residues involved in C1q binding are depicted in yellow[34–36]. **C.** Residue E430 (enhancing amino acid) is shown in blue[11]. Residue K439 (charge repulsion amino acid) is indicated in red[11]. **D.** Interaction of the Fc-binding peptide[12] (shown in red) with the Fc domain of IgG (pdb 1DN2: Fc domain docked to IgG1-b12 Fc domain, 1DN2 Fc domain hidden).

Results

Disruption of IgG Fc-Fc contacts interferes with complement activation on the bacterial surface

To examine whether clustering of IgG molecules occurs on bacteria, we made use of an inhibitory peptide that disrupts Fc-Fc contacts[12]. This Fc-binding peptide binds to residues of the IgG Fc domain that are involved in Fc-Fc interactions during antibody hexamerization[11,12] (**figure 1D**). Previously, it was shown that the Fc-binding peptide inhibits complement activation on tumor cells, thereby blocking complement dependent cytotoxicity (CDC)[11]. Here, we investigated whether this peptide affects the deposition of complement factors on *S. aureus*. First, we analyzed whether the Fc-binding peptide influences C4b deposition, since C4b is the first complement component covalently deposited on the bacterial surface by C1. Fluorescently labeled *S. aureus* strain Wood 46 was incubated with normal human serum (NHS), as a source of complement and naturally occurring antibodies against *S. aureus*[13,14] in the absence or presence of the Fc-binding peptide or a control peptide. Flow cytometry analyses showed that incubation of *S. aureus* with serum resulted in significant levels of C4b deposition on the bacterial surface (**figure 2A**). The decrease in C4b deposition at serum concentrations above 1% could be due to high quantities of other complement components deposited via the alternative pathway, which is the predominant pathway at higher serum concentrations. However, when the Fc-binding peptide was added during opsonization of *S. aureus*, the levels of deposited C4b were strongly decreased at all tested serum concentrations (**figure 2A**). C4b levels in the presence of the Fc-binding peptide were even comparable to those of the heat-inactivated (HI) serum control (no active complement), while the control peptide did not interfere with C4b deposition. To also examine the influence of the Fc-binding peptide on the deposition of C3b molecules, we performed the same incubations but now used an anti-C3 antibody for detection. In the presence of the inhibitory peptide, C3b deposition on *S. aureus* was also significantly inhibited at low serum concentrations (< 2.5%) (**figure 2B**). At higher serum concentrations (>2.5%) the Fc-binding peptide did not influence C3b deposition, which could be due to a) the deposition of C3b molecules on the bacterial surface through other complement pathways, b) the high density of antibodies at these serum concentrations which would diminish the necessity for Fc-Fc interactions to form IgG hexamers, or c) the peptide concentration is simply insufficient for interaction with all IgG at these serum levels, however the molar ratio indicates this should be sufficient (approx. 4µM IgG in 5% serum to 13µM peptide). Strikingly, the binding of C1q was not inhibited by the Fc-binding peptide and even seemed to increase in its presence (**Supplemental figure 1**). Nevertheless, our C4b and C3b deposition assays show that the binding of C1 in the presence of the Fc-binding peptide did not lead to activation of the complement cascade. To study the dose-dependent inhibitory effect of the Fc-binding peptide on C4b and C3b deposition, we incubated *S. aureus* with a fixed concentration of serum (1%) and a concentration range of the Fc-binding peptide. As extra controls we used two peptides that are scrambled versions of the Fc-binding peptide sequence and therefore more closely related to the active peptide than the previously used control peptide. Both C4b and C3b deposition were inhibited by the Fc-binding peptide in a dose-dependent

manner (**figure 2C and 2D**), while the scrambled peptides showed no inhibitory effects. Overall, these data show that naturally occurring antibodies in human sera require Fc-Fc contacts to induce classical pathway activation on *S. aureus*.

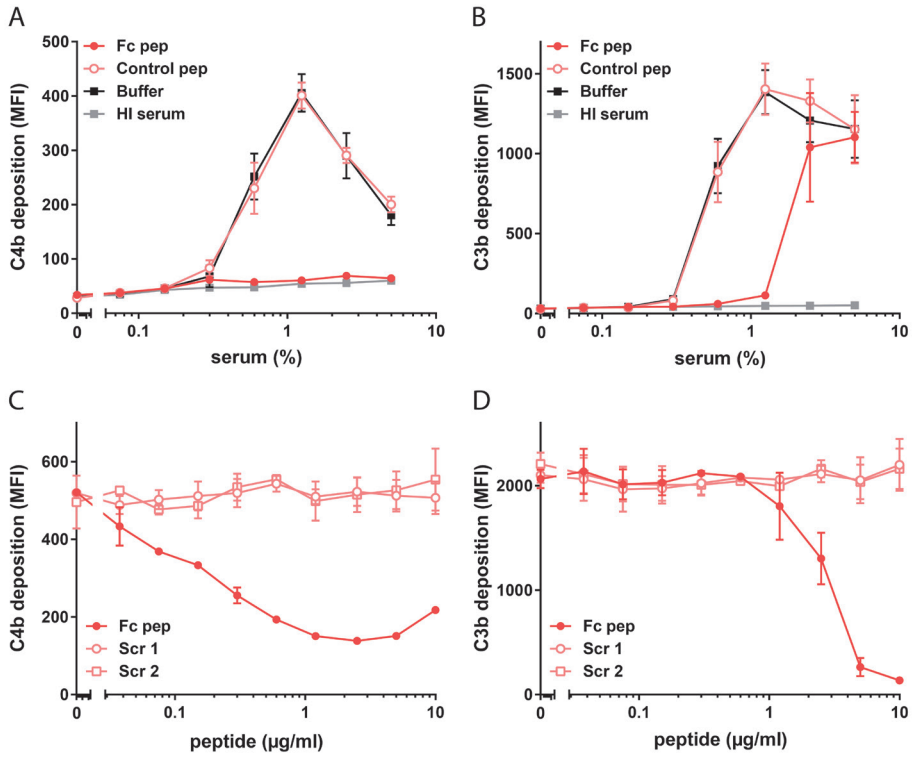


Figure 2. Disruption of IgG Fc-Fc contacts interferes with complement activation on the bacterial surface. A-D. Complement deposition on *S. aureus* strain Wood 46 with normal human serum, in the presence of the Fc-binding peptide (Fc pep) or control peptides, measured by flow cytometry. Detection with a monoclonal anti-human C4d or C3d antibody (1 µg/ml). **A-B.** Set concentration of Fc-binding and control peptide (both 20 µg/ml) used. **A.** Analysis of C4b deposition, showing inhibition when hexamer formation is disturbed. **B.** Analysis of C3b deposition, also showing a decrease in deposition in the presence of the Fc-binding peptide. **C-D.** Set concentration of serum (1%) used and a concentration range of the Fc-binding peptide or two scrambled versions of the Fc-binding peptide sequence (Scr 1 and Scr 2). **C.** Analysis of C4b deposition, showing a dose-response of the inhibitory peptide and no effect of the scrambled peptides. **D.** Analysis of C3b deposition, also showing a decrease in C3b deposition in the presence of the inhibitory peptide in a dose-dependent manner. All figures represent the mean ±se of three separate experiments.

Disruption of antibody Fc-Fc contacts blocks phagocytosis and C5a release

Since the deposition of both C4b and C3b was inhibited by the Fc-binding peptide, we wondered whether the eventual phagocytic uptake of the bacteria would be influenced as well. Therefore, we incubated fluorescently labeled Wood 46 with NHS in the presence of the Fc-binding peptide, after which human neutrophils were added. Phagocytosis was quantified after 15 minutes by measuring the fluorescence of gated neutrophils by flow cytometry. In the presence of serum, fluorescent bacteria were taken up by neutrophils,

in a dose-dependent manner (**figure 3A**). In contrast to the control peptide, the Fc-binding peptide potently blocked phagocytosis at all tested peptide concentrations. Again we observed that the level of inhibition was influenced by the percentage of serum used. At 10% serum none of the tested the Fc-binding peptide concentrations was able to inhibit phagocytosis, which again could be explained by the high level of antibodies at this concentration. Since the inhibition of phagocytosis by the peptide was almost complete at serum concentrations of 1% and less, this suggests that the classical pathway of complement is crucial for phagocytosis at these concentrations of serum. To confirm that the final steps of the complement cascade and formation of the important chemoattractant C5a were also influenced by antibody multimerization, we quantified released C5a products. Therefore, Wood 46 was incubated with NHS in the presence of inhibitory peptide. Following incubation for 30 minutes, the bacteria were centrifuged and supernatant was collected for C5a analyses (as described previously[15]). We observed that the Fc-binding peptide inhibited the formation of released C5a products (**figure 3B**). Altogether, we show that interference with IgG Fc-Fc contacts on *S. aureus* diminishes bacterial phagocytosis by neutrophils and the formation of chemoattractant C5a.

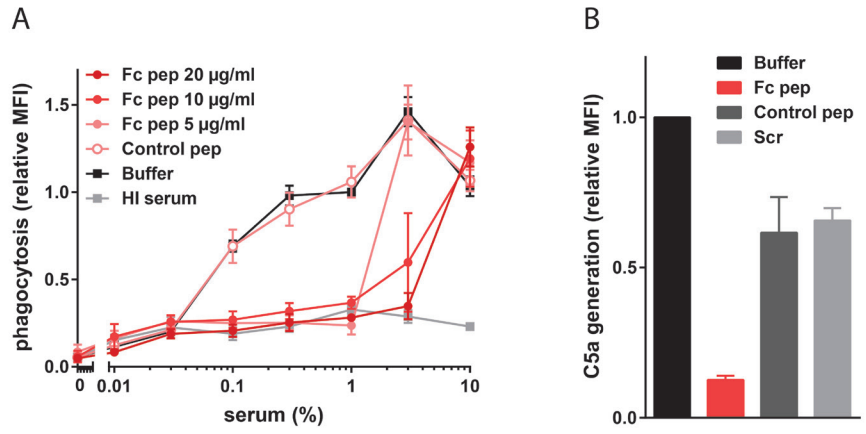


Figure 3. Disruption of antibody Fc-Fc contacts blocks phagocytosis and C5a release. A. Phagocytosis of FITC-labeled Wood 46 by human neutrophils in the presence of normal human serum and the Fc-binding peptide (Fc pep; 5, 10 or 20 µg/ml), measured by fluorescence (geomean) of the neutrophils. **B.** C5a generation of supernatants of *S. aureus* strain Wood 46 incubated with serum (3%) and Fc-binding or control peptides (20 µg/ml), measured by Ca^{2+} mobilization of C5aR-U937 cells, displayed by the relative fluorescence (compared to the buffer condition). Both figures represent the mean ±se of three separate experiments.

Monoclonal antibodies against wall teichoic acid can be modified for enhanced complement activation

Next, we studied whether we could engineer human monoclonal antibodies to enhance complement activity on *S. aureus.* First, we selected human IgG1 molecules directed against two *S. aureus* surface epitopes and introduced Fc interface mutations that would increase hexamer assembly[11]. We produced recombinant human IgG1 against wall teichoic acid (WTA)[4] and Clumping factor A (ClfA) (**figure 4A**). Wild-type antibodies (lacking Fc

modifications) were first analyzed for their ability to bind several *S. aureus* strains by flow cytometry (**figure 4B**). Since anti-WTA antibodies showed superior binding to all tested *S. aureus* compared to anti-ClfA, we selected the anti-WTA antibody for further mutational analyses. In addition to a mutant for enhanced hexamerization (Enhancing variant E430G), we also created two charge repulsion mutants that are weakened in their Fc-Fc interactions (Inhibiting variants K439E and S440K) (**figure 1C**). To make sure antibody binding itself was not influenced by the mutations in the Fc domain, we verified that binding of the mutants and the wild type (WT) antibody to *S. aureus* strain Wood 46 and USA300 was equal (**figure 4C**). Then, we investigated whether these recombinant antibodies could influence the phagocytosis of *S. aureus* by human neutrophils. First, we observed that addition of anti-WTA antibody enhances uptake of *S. aureus* in a dose-dependent manner (**figure 4D and 4E**). This increase could be observed serum devoid of natural antibodies (**figure 4D**) and in normal human serum (**figure 4E**). Importantly, we observed that phagocytosis by the hexamer enhancing E430G antibody was significantly higher than by the WT antibody (**figure 4D and 4E**). Differences between E430G and WT antibody were specifically observed at lower concentrations of IgG, but were not measurable at higher IgG levels, where phagocytosis levels reached their maximum (**figure 4D, E**). The control isotype IgG (IgG1 b12) did not induce phagocytosis. Since naturally occurring antibodies against WTA predominantly include IgG2[14], we also engineered recombinant human IgG2 against WTA and used these antibodies in our phagocytosis assay. Also for IgG2 the E430G variant showed higher levels of phagocytosis than the WT antibody (**supplemental figure 2**). Notably, in the absence of serum the IgG1 antibody was able to induce more phagocytic uptake than the IgG2 antibody. However, addition of serum and thus complement had a stronger effect with the IgG2 antibody than with IgG1, which does not support the hypothesis that the IgG2 subclass is inefficient in activating complement[16,17]. Next, we analyzed the capacity of the IgG1 antibodies to deposit C4b and C3b in a purified classical pathway system. Purified components were used instead of serum to guarantee that natural antibodies against *S. aureus* were not present and could therefore not interfere with our measurements of hexamer variation. *S. aureus* Wood 46 was incubated with antibodies and subsequently with purified C1 and C4. In the presence of the WT antibody, C4b deposition was observed in a dose-dependent manner (**figure 4F**). Reduced C4b deposition was observed for the charge repulsion mutants K439E or S440K. Since the introduced charge repulsion should be reversed when these two mutants are mixed, we also studied C4b deposition by a mixture of these two antibodies and found that C4b deposition was restored back to WT. Notably, the presence of the hexamer enhancing variant E430G showed a substantial increase in C4b deposition. Next, we used the same assay system to measure C3b deposition by these antibody variants. Therefore an additional incubation step with C2 (to create C4b2a) and C3 was allowed to deposit C3b. We observed similar results to the C4b deposition; levels of C3b deposition could be inhibited by the K439E or S440K variants but, more importantly, could also be enhanced by the anti-WTA E430G variant (**figure 4G**). Overall, these results indicate that complement deposition and subsequent phagocytosis of *S. aureus* can be strengthened by IgG molecules with enhanced Fc-Fc contacts.

Monoclonal antibodies against capsular polysaccharides can be modified for enhanced complement activation

Since about 75% of *S. aureus* clinical strains expresses a polysaccharide capsule[18] that can shield surface molecules (like WTA) from recognition by antibodies, we also designed modified IgG1 antibodies against capsular polysaccharide 5 (**figure 5A**) and tested binding of these monoclonal antibodies to several CP5-encapsulated *S. aureus* strains (**figure 5B**). This showed strong binding of the antibody to strain Reynolds CP5, known to express high levels of CP5. We observed less antibody binding to strains COL and Newman$^{-/-}$, which is likely due to lower levels of CP5 expression in these strains compared to Reynolds CP5[19,20]. We selected the high CP5-expressing strain Reynolds for further analysis. Again, we verified that the binding of the IgG variants to bacteria was not affected by the modifications in the Fc domain (**figure 5C**). Next, we investigated whether phagocytosis was influenced by the modification of the monoclonal CP5 antibody. Since serum of healthy individuals does not contain high concentrations of naturally occurring antibodies against capsular polysaccharides[19,21], all experiments were performed in the presence of NHS. GFP-labeled Reynolds CP5 was incubated with the CP5 WT antibody and E430G variant and subsequently serum and neutrophils were added. As shown previously (see Chapter 2 and[22]), we observed that addition of anti-CP5 antibodies can counteract the anti-phagocytic activity of the capsule. Furthermore, an increase of phagocytosis was observed with the E430G variant compared to the WT antibody (**figure 5D**). To verify the effect of the Fc-binding peptide on IgG multimerization and therefore the influence on phagocytosis, we added the peptide and a scrambled version to the phagocytosis assay. The Fc-binding peptide was able to potently inhibit phagocytosis by both the WT and E430G anti-CP5 antibody (**figure 5E**). Next, we analyzed the effect of these antibodies on C4b and C3b deposition in serum (**figure 5F and 5G**). Strain Reynolds CP5 was incubated with the monoclonal CP5 antibodies and NHS. Both C4b and C3b deposition were enhanced with the E430G variant compared to the WT antibody, although for C4b deposition this effect was observed at low concentrations of IgG while only at higher IgG concentrations, C3b deposition could be enhanced (**figure 5G**). Overall, these results indicate that hexamer formation of antibodies directed against capsular polysaccharides can be increased and that this has a strong positive influence on complement deposition and phagocytosis of encapsulated *S. aureus* strains.

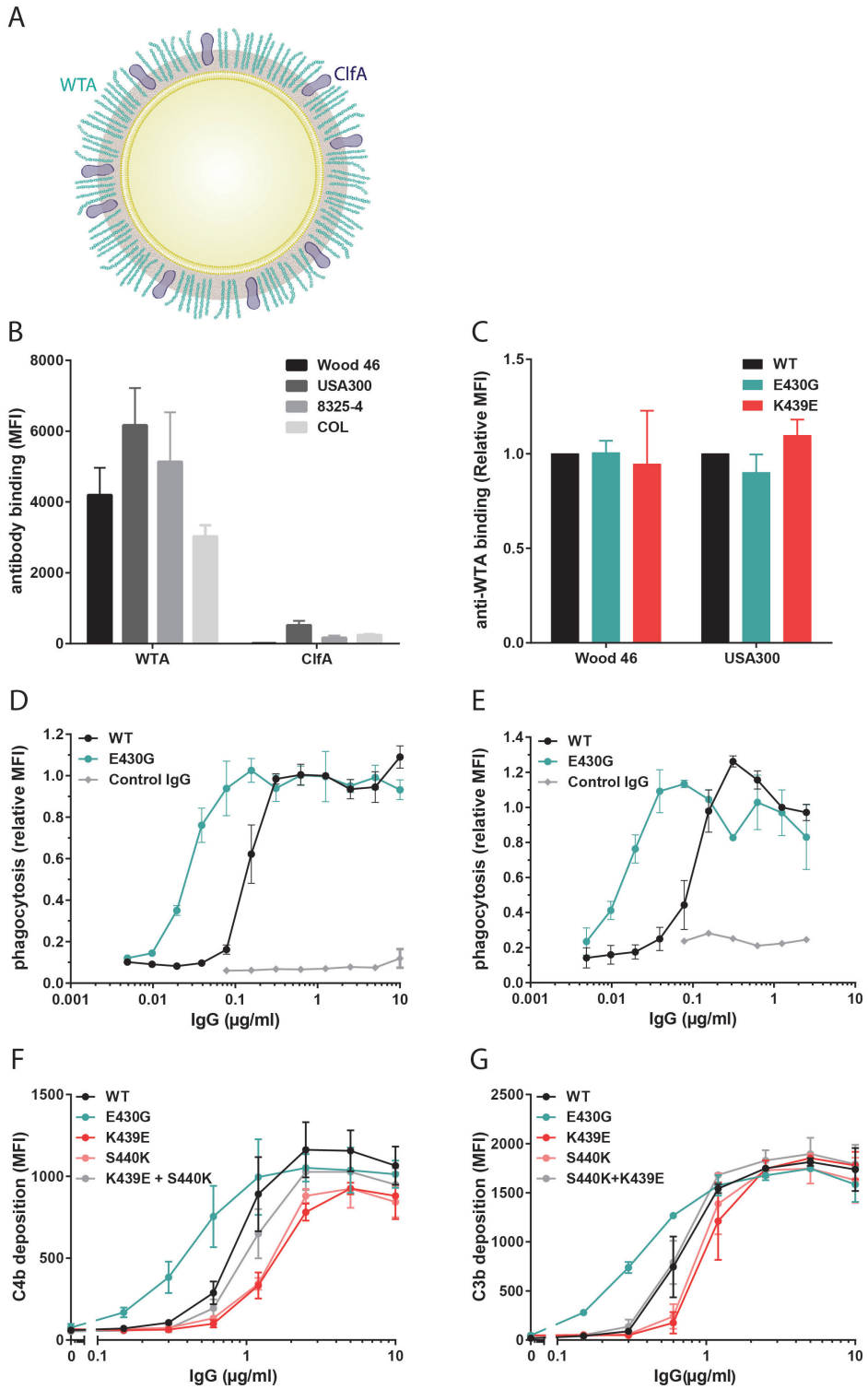
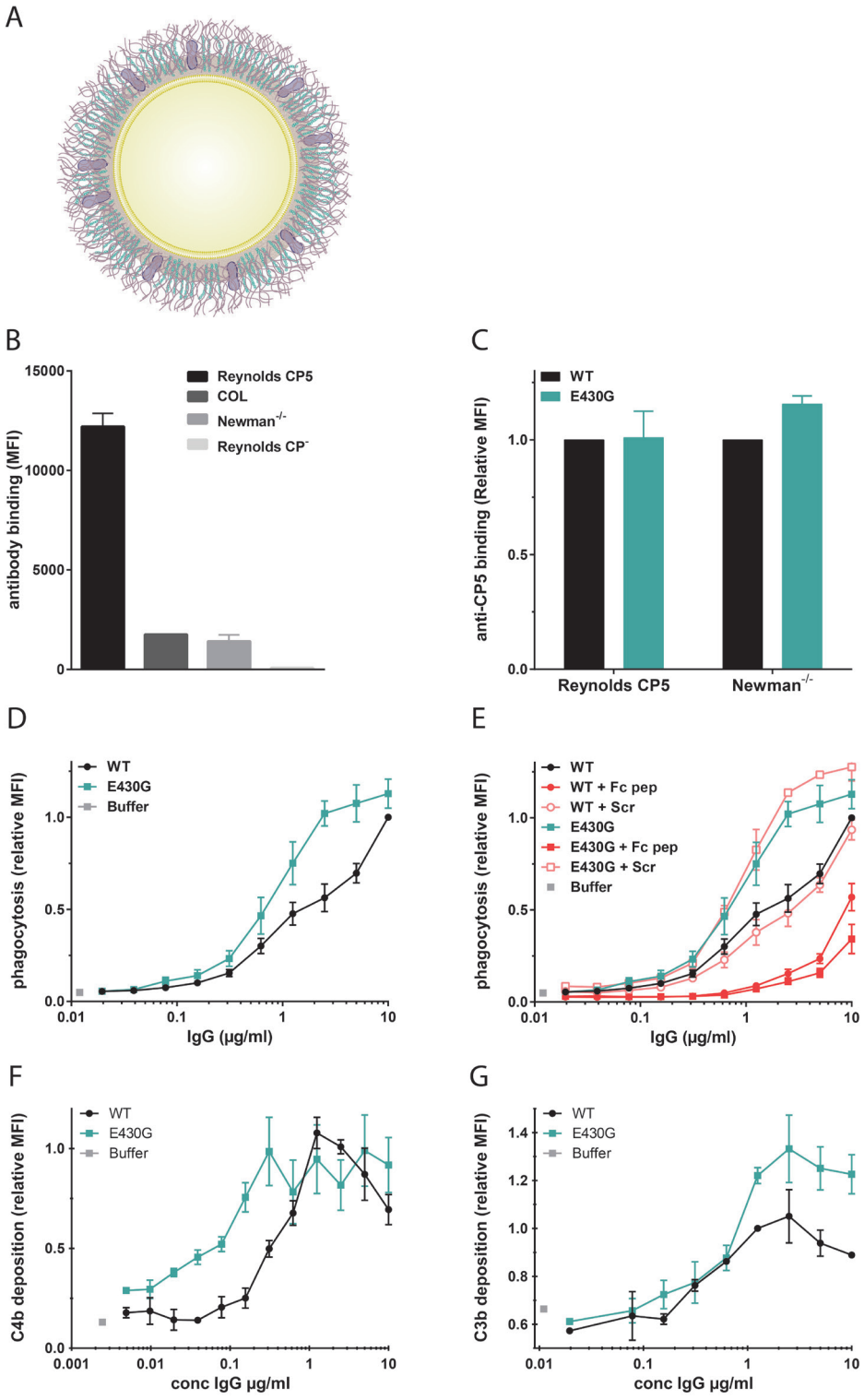


Figure 4. Monoclonal antibodies against wall teichoic acid can be modified for enhanced complement activation. A. Schematic representation of *S. aureus,* showing the location of WTA and ClfA, attached to the peptidoglycan in the bacterial cell wall. **B-C.** Binding of monoclonal antibodies (1 µg/ml) to the surface of *S. aureus*, detected with a monoclonal antibody against human IgG (1 µg/ml) by flow cytometry. **B.** Binding of WT WTA and ClfA monoclonal antibodies to several *S. aureus* strains, showing strong binding for WTA and low binding for ClfA on all tested strains. **C.** Binding of WTA WT antibody or the hexamer enhancing (E430G) or inhibiting (K439E and S440K) variant to Wood 46 and USA300, showing comparable binding levels for all variants, as displayed by the relative fluorescence (compared to the WT on each strain). **D-E.** Phagocytosis by human neutrophils of GFP-labeled *S. aureus* Wood 46 with serum and a concentration range of the WTA monoclonal antibodies. This was measured by flow cytometry as the fluorescence of the neutrophils and displayed by the relative fluorescence (compared to the WT at 1.25 µg/ml). **D.** Phagocytosis in the presence of IgG/IgM depleted serum (3%), showing increased uptake with the E430G variant of the antibody. **E.** Phagocytosis in the presence of NHS (0.1%), also showing an enhanced effect with the E430G variant. **F-G.** Complement deposition on *S. aureus* Wood 46 with purified components and a concentration range of the WTA monoclonal antibodies, detected with a monoclonal anti-human C4d or C3d antibody (both 1 µg/ml) and measured by flow cytometry. **F.** C4b deposition, showing enhancement with the E430G variant and a decrease of deposition with the K439E and S440K variant. **G.** C3b deposition, also showing increased deposition with the E430G variant and inhibition with the K439E and S440K variant. All figures represent the mean ±se of three separate experiments.

Figure 5. Monoclonal antibodies against capsular polysaccharides can be modified for enhanced complement activation. A. Schematic representation of *S. aureus* showing the polysaccharide capsule covering the bacterial surface. **B-C.** Binding of WT CP5 monoclonal antibodies (1 µg/ml) to the surface of several *S. aureus* strains, detected with a monoclonal antibody against human IgG (1 µg/ml) by flow cytometry. **B.** Binding of WT CP5 monoclonal antibodies to several encapsulated *S. aureus* strains, showing very strong binding to the Reynolds CP5 strain compared to the other strains. **C.** Binding of CP5 WT antibody and the hexamer enhancing (E430G) variant to *S. aureus* strain Reynolds CP5 and Newman$^{-/-}$, showing comparable binding levels for both variants, as displayed by the relative fluorescence (compared to the WT of each strain). **D-E.** Phagocytosis by human neutrophils of GFP-labeled *S. aureus* Reynolds CP5 with serum (3%) and a concentration range of the CP5 monoclonal antibodies, measured by flow cytometry as the fluorescence of the neutrophils, as displayed by the relative fluorescence (compared to the WT at 10 µg/ml). **D.** Analysis of phagocytosis, showing an enhancing effect with the E430G variant. **E.** Phagocytosis in the presence of the monoclonal antibodies and the Fc-binding peptide (Fc pep) or a scrambled peptide (Scr), both 10 µg/ml, showing that the inhibitory effect of the Fc-binding peptide disrupts hexamer formation of both the WT and E430G variant of the antibody. **F-G.** Complement deposition on *S. aureus* Reynolds CP5 with serum or HI serum (both 3%) and a concentration range of CP5 antibodies, detected with a monoclonal anti-human C4d or C3d antibody (both 1 µg/ml) and measured by flow cytometry. **F.** C4b deposition, showing the enhancing effect of the E430G variant. **G.** C3b deposition, also showing increased deposition in the presence of the E430G variant. All figures represent the mean ±se of three separate experiments.



Discussion

In this paper we demonstrate that Fc-Fc contacts between IgG molecules are crucial for proper activation of the classical complement cascade on *S. aureus*. Diebolder *et al.* showed for the first time that IgG molecules form ordered complexes via non-covalent interactions between Fc regions. Those studies, performed on tumor cells using engineered monoclonal antibodies, showed that multimeric IgG molecules recruit C1q to trigger effective MAC-dependent killing of tumor cells (complement-dependent cytotoxicity (CDC)). In this paper we provide evidence that also naturally occurring human antibodies arrange into highly organized structures on bacteria. Using an inhibitory peptide that disrupts Fc-Fc interactions, we find that thesecontacts between naturally occurring IgG molecules are important for effective deposition of complement components C4b and C3b onto bacteria and the subsequent biological events such as generation of the chemoattractant C5a and phagocytic uptake of the bacteria by neutrophils. Furthermore, our studies indicate that the effectiveness of monoclonal antibodies recognizing *S. aureus* surface antigens can be enhanced by mutations in the Fc region that favor multimerization. Since an IgG hexamer is the most optimal form for complement activation[11], we believe that Fc-Fc contacts favor IgG hexamerization on bacteria. However, since the exact arrangement of IgG molecules on the bacterial surface could not be demonstrated, we cannot exclude that other multimeric arrangements (trimer, tetramers, pentamers etc.) may be formed. In line with studies on tumor cells, we show here that complement activity on *S. aureus* is facilitated by interactions between specific Fc regions of neighboring IgG molecules. Interestingly, our C1q binding assay indicated that the Fc-binding peptide could not interfere with binding of C1q to the bacterial surface. This could indicate that multivalent binding of C1q to hexameric IgG might be required for proper activation of C1qrs. The catalytic regions of the C1r molecules are positioned at opposite ends inside the C1 complex[23]. It was suggested that upon ligand binding, conformational changes in the flexible hinge regions of C1q create the space needed for the C1r regions to make contact and activate the molecule. This will lead to the activation of C1s and subsequent initiation of the classical pathway cascade. It is possible that the presence of Fc-binding peptide still allows binding of C1 to non-hexameric IgG molecules but that this monovalent binding will not lead to activation of the attached serine proteases. This is supported by the fact that the binding site for C1q on IgG is in a different location than the binding site for the Fc-binding peptide and thus simultaneous binding of both molecules should be possible. IgG harbors two binding sites for C1q, one on each Fc C_H2 domain. It was recently described that when IgG molecules arrange into a hexamer, only one Fab domain is bound to a target while the other is detached from the cell surface. Potentially this shows the need for two C1q binding regions since only one will be available for C1 assembly in the hexameric formation[24]. Next to the importance of Fc-Fc contacts, our results with the Fc-binding peptide in human immune sera also seem to indicate that the classical pathway is the major pathway on *S. aureus* at the serum concentrations used in our assays. This is confirmed by the observation that C4b deposition is completely inhibited in the presence of this peptide and is supporting the hypothesis that the involvement of the lectin pathway is trivial in activating complement on *S. aureus* in healthy adults[25].

Monoclonal antibodies with increased complement activity on *S. aureus* could serve as an attractive therapy for treatment or prevention of *S. aureus* infections in the hospital. Modification of monoclonal antibodies in the Fc region could enhance complement-dependent clearance of bacteria via more effective recruitment of neutrophils and enhanced phagocytosis rates. It is clear from our study that the success of such an approach will be highly dependent on the choice of the antibody epitope. In contrast to the antibodies against ClfA, antibodies directed against the abundant wall teichoic acids showed very effective complement-dependent phagocytosis of *S. aureus*. Although, the presence of naturally occurring anti-WTA antibodies in patients might reduce the therapeutic effectiveness, we were able to measure the influence of the WTA antibody variants at low concentrations of NHS. Presumably, a combination of modified monoclonal antibodies targeting multiple antigens on the bacterial surface would provide the most effective treatment in patients with different antibody repertoires and will target *S. aureus* strains with different genetic backgrounds. Since our study involves only *in vitro* experiments so far, we are not yet able to predict the precise influence of enhancing hexamerization on bacteria *in vivo*. Furthermore, applying the phagocytosis-enhancing effect of our antibody modifications to the recent findings of anti-WTA-antibiotic drug conjugates[4] could potentially be used to further enhance clearance of intracellular bacteria. Additionally, the generation of C5a is essential for recruitment of phagocytic cells to the site of infection and thus enhancing this will contribute further to clearing *S. aureus* from the body.

Overall, in this study we show for the first time that antibody molecules arrange into hexamers on *S. aureus*. Finally, the methods used here allow us to produce engineered antibodies against staphylococcal surface antigens, and thus presumably these enhancing effects on classical pathway activation can be accomplished on other pathogenic bacteria as well.

Materials & Methods

Bacterial strains and fluorescent labeling

S. aureus strains Wood 46, USA300, 8325-4, COL, Newman spa^{-}/sbi^{-} (Newman$^{-/-}$), Reynolds CP5 and Reynolds CP^{-} [20] were used in this study. As indicated, strains were fluorescently labeled with either fluorescein isothiocyanate (FITC) or Green Fluorescent Protein (GFP). For generation of FITC-labeled *S. aureus,* bacteria were grown in Todd Hewitt Broth at 37°C overnight, washed and resuspended in PBS. FITC (0.5 mg/ml in DMSO) was added and incubated for 30 min on ice. Bacteria were washed twice, resuspended in RPMI-0.05% HSA and stored at -20°C. GFP-labeled bacteria were prepared by transformation with the pCM29 plasmid, constitutively expressing GFP under regulation of the sarA promoter as previously described[26,27]. Reynolds and encapsulated control strains were grown on Columbia agar (Oxoid) supplemented with 2% NaCl (CSA) for 24 hours at 37°C to guarantee optimal expression of the polysaccharide capsule[19,28].

Isolation of human serum and neutrophils

For preparation of serum, venous blood was collected from healthy volunteers in 9 ml vacutainer blood tubes containing a clot activator (BD). Clotting was allowed for 15 minutes and serum was collected by centrifugation at 2080g for 20 min at 4°C. Sera of 20 healthy volunteers was pooled and stored at -80°C. Heat-inactivated (HI) serum was prepared by incubation at 56°C for 30 min. For IgG/IgM depletion of NHS, 5 mM EDTA was added and 5 ml serum run over a 5 ml HiTrap Protein G column (GE healthcare) in tandem with a 5 ml HiTrap NHS-Sepharose column coupled with goat-anti-Hu-IgM in the cold with 20 mM sodium phosphate buffer pH 7.5. Collected peak fractions were pooled, reconstituted with 10 mM $CaCl_2$ + 10 mM $MgCl_2$, and stored again at -80°C. For the isolation of human neutrophils, blood from a healthy donor was collected in heparin vacutainers (BD) and cells were isolated using the Ficoll-Histopaque gradient method[29]. Cells were used on the same day.

Expression constructs for antibodies

For antibody expression variable heavy (VH) chain and variable light (VL) chain sequences were cloned in pcDNA3.3 expression vectors containing human IgG1 heavy chain (HC) and light chain (LC) constant regions. Desired mutations were introduced either by gene synthesis or site directed mutagenesis. Anti-MRSA Antibodies mentioned in this application have VH and VL sequences derived from previously described antibodies anti-wall teichoid acid GlcNAc beta 4497 (anti-WTA-4497; based on WO2014/193722[4]), anti-ClfA tefibazumab (based on WO2002/072600) and anti-capsular polysaccharide type 5 (anti-CP5-137G18A; based on WO2014/027698). In some of the examples the human IgG1 antibody b12, a gp120-specific antibody was used as a negative control[30]. Mutations were introduced in the Fc domain as previously described, creating antibodies modified to form enhanced hexamers (E430G mutation)[31] or weakened hexamers (S440K or K439E mutations)[11].

Transient expression

Antibodies were expressed as IgG1,κ. Plasmid DNA mixtures encoding both heavy and light chains of antibodies were transiently transfected in Expi293T cells (Life technologies, USA) using 293fectin (Life technologies) essentially as described by Vink *et al.*[32].

Purification and analysis of proteins

Antibodies were purified by affinity chromatography (rProtein A FF; GE Healthcare), as previously described[11]. Concentration of the antibodies was determined by absorbance at 280nm. Batches were analyzed by a number of bioanalytical assays including SDS-PAGE, size exclusion chromatography and measurement of endotoxin levels.

Peptides

The Fc-binding peptide (DCAWHLGELVWCT)[12], scrambled versions of the Fc-binding peptide sequence (Scr 1: ACWTLEWGVLDCH; Scr 2: WCDLEGVTWHACL) and control peptide (GWTVFQKRLDGSV) were synthesized by Pepscan (Lelystad, The Netherlands) and solved in MilliQ water.

Antibody binding assay

Strains were grown overnight on sheep blood agar (SBA) at 37°C or were cultured overnight in Todd Hewitt Broth (THB) and subsequently sub cultured in fresh THB for 3h. For anti-CP5 detection, strains were grown on CSA for 24h at 37°C. Bacteria were suspended in PBS, washed and resuspended in HEPES buffer (20 mM HEPES, 140 mM NaCl) containing 5 mM $CaCl_2$ and 2.5 mM $MgCl_2$ ($HEPES^{++}$) with 0.5% bovine serum albumin (BSA) or RPMI containing 0.05% human serum albumin (HSA). To determine the binding capacity, antibodies against WTA, ClfA or CP5 (all 1 µg/ml) were added and incubated for 30 min at 4°C, shaking (600 rpm). Bacteria were washed twice with PBS containing 1% BSA (PBS-BSA) or RPMI-HSA and incubated with an APC-conjugated polyclonal anti-human IgG antibody ($F(ab')_2$, Jackson, 1:350) for 45 min at 4°C, shaking ((±700 rpm). Samples were washed, fixed with cold paraformaldehyde (PFA, 1%) and analyzed using flow cytometry (FACS Verse, BD).

Complement deposition assays

Strains were grown overnight on SBA at 37°C or on CSA for 24h at 37°C. Bacteria were suspended in PBS, washed and resuspended in $HEPES^{++}$-BSA. For the complement deposition assays with normal human serum (NHS), a pre-incubation of serum with buffer, peptides (Fc-binding peptide or control peptide, 20 µg/ml) or CP5 monoclonal antibodies (IgG1-CP5-137G18A-F405L WT or E430G variant) for 10 min at RT, was performed and added to washed bacteria ($5*10^7$/ml) for 20 min at 37°C, shaking (±700 rpm). As an additional control HI serum was used, in which complement will not be activated. For the complement deposition assay with purified components, WTA antibodies (IgG1-WTA-S4497 WT, E430G, S440K or K439E variant) were added to bacteria ($5*10^8$/ml) and incubated for 30 min at 4°C, shaking (600 rpm). Bacteria were washed twice with PBS-BSA and incubated with C1 (1 µg/ml, Complement Technology) for 30 min at 4°C, shaking (±700 rpm). After another washing step, samples were incubated with polyclonal anti-human C1q antibody (1 µg/ml, Dako) for C1q binding, or C4 and, for C3b deposition, subsequently with C2 together with C3 (all 10 µg/ml, Complement Technology) for 30 min at 37°C, shaking (±700 rpm). For both serum and purified component assays bacteria were then washed twice with PBS-BSA and incubated with either a monoclonal anti-human C4d or C3d antibody (both 1 µg/ml, Quidel) for 45 min at 4°C, shaking (±700 rpm). After another washing step, an incubation with a FITC- or APC-conjugated polyclonal anti-mouse Immunoglobulins antibody (1 µg/ml, Dako or BD, respectively) was performed for 45 min at 4°C, shaking (±700 rpm). Samples were washed, fixed with cold PFA (1%) and analyzed by flow cytometry.

Phagocytosis assays

Fluorescently labeled *S. aureus* ($5*10^7$/ml) was pre-incubated with NHS or IgG/IgM depleted serum in RPMI-HSA in the presence or absence of peptides (Fc-binding peptide, control peptide or scrambled peptides, 5, 10 or 20 µg/ml) or monoclonal antibodies for 15 min at 37°C. Freshly isolated human neutrophils ($5*10^6$/ml) were added and phagocytosis was allowed for 15 min at 37°C, shaking (±700 rpm). Cold PFA (1%) in RPMI-HSA was added to stop the reaction and samples were analyzed for fluorescence of the neutrophils by flow cytometry.

Calcium mobilization assay

Supernatants of bacteria incubated with serum (3%) and Fc-binding peptide or control peptides (20 µg/ml) for 15 min at 37°C, were isolated and added as a stimulus to U937-C5aR cells as described previously[15]. In short, U937-C5aR cells were labeled with 2 µM Fluo-3-AM. Increase of calcium mobilization was detected as an increase in fluorescence by flow cytometry and was measured 9 seconds before and 40 seconds after stimulation with the supernatants. As a positive control, C5a (10^{-8} M, Bachem) was added.

Acknowledgements

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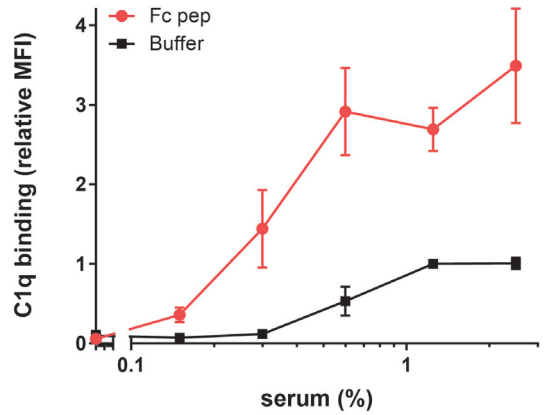
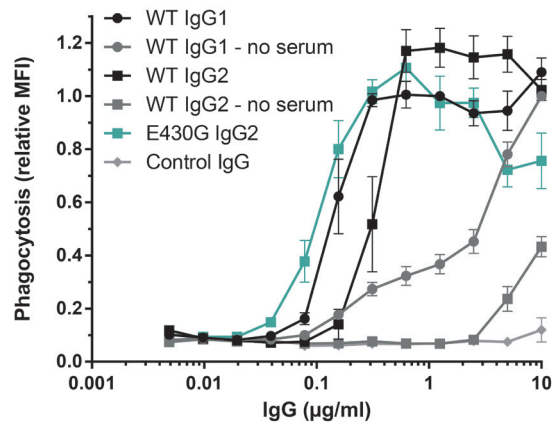
Supplemental information



Supplemental figure 1. C1q binding to *S. aureus* in the presence of serum and the Fc-binding peptide (Fc pep; 20 µg/ml), detected with a FITC-conjugated polyclonal anti-human C1q antibody, showing an increase in C1q binding in the presence of the Fc-binding peptide. This was measured by flow cytometry and displayed as the relative fluorescence (compared to Buffer at 1.25%). Graph represents the mean ±se of two separate experiments.



Supplemental figure 2. Phagocytosis of GFP-labeled Reynolds CP5 by human neutrophils in the presence and absence of IgG/IgM depleted serum (3%) and monoclonal IgG1 and IgG2 WTA antibodies, measured by flow cytometry as the fluorescence of the neutrophils, showing a stronger increase of phagocytosis by the presence of complement with the WT IgG2 antibody than with the WT IgG1 antibody, as displayed by the relative fluorescence (compared to the WT IgG1 at 1.25 µg/ml). Graph represents the mean ±se of three separate experiments.

Chapter 5

Antibody hexamerization improves Fc receptor-mediated phagocytosis of *Staphylococcus aureus*

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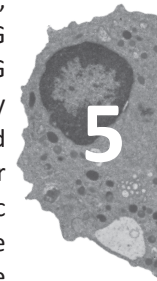
Abstract

The uptake of *Staphylococcus aureus* by phagocytic cells is crucial for the clearance of the pathogen from the body. This process is dependent on the opsonization of the bacterial surface with both antibodies and complement factors. Previously, it was suggested that the formation of multimeric IgG, mediated through Fc-Fc interactions, at the surface of tumor cells as well as *S. aureus* is essential for the effective activation of the complement system. Now, we find that this arrangement of antibodies on the bacterial surface is also important for Fc receptor-mediated phagocytosis of *S. aureus*. Through monoclonal antibodies targeting *S. aureus* wall teichoic acid, engineered to enhance intermolecular Fc-Fc interactions, we are able to enhance the Fc-mediated phagocytic uptake of *S. aureus*. These findings could be important in understanding the interactions of surface-associated antibodies with neutrophil Fc receptors and for the development of effective monoclonal antibodies for treatment of *S. aureus* infections.

Introduction

Neutrophils are essential cells in the first line of defense against invading pathogens. They are the predominant leukocytes in human blood, are very rapid responders and have various ways in which they can recognize bacteria, engulf them and subsequently kill them[1–3]. Bacterial recognition is mediated through direct interaction with microbial surface structures or via opsonins associated with the bacterial surface. Efficient opsonization will occur through the deposition of complement factors and the binding of specific antibodies to bacterial surface structures. This leads to recognition by neutrophil complement receptors and Fc receptors, respectively. Eventually, activation of the cell will trigger several intrinsic signaling pathways inducing phagocytosis and subsequent killing of the bacteria[4]. Next to their direct opsonic capacity, antibodies activate the classical pathway of complement by binding and activating C1[5]. Recently, Diebolder *et al* described that specific arrangement of surface-associated antibodies into hexameric formations via Fc-Fc interactions (**figure 1A**) is crucial for efficient activation of the complement cascade via the classical pathway[6]. Through specific mutations in the Fc domain of monoclonal antibodies they showed that complement-dependent lysis of tumor cells is influenced by enhanced or inhibited Fc-Fc interactions. Additionally, we showed that these Fc-Fc arrangements are critical for activation of the complement system on bacterial surfaces, specifically *Staphylococcus aureus* (*S. aureus*), and for subsequent phagocytic uptake by neutrophils (Chapter 4). It was previously suggested that activation of the cell through both Fc receptors as well as complement receptors is crucial for effective phagocytosis[7,8]. In this study, we therefore focus on the effect of Fc-Fc contacts at the staphylococcal surface on the direct interaction of antibodies with Fc receptors (**figure 1B**) and subsequent Fc receptor-mediated phagocytosis. The most abundant and important activating Fc receptor expressed on human neutrophils is Fc gamma receptor (FcγR)IIa, followed by FcγRIIIb. Both receptors are primarily capable of binding Immunoglobulin G (IgG), only when aggregated in complex with antigens[4,9]. The different subclasses of IgG (IgG1, IgG2, IgG3, IgG4, in order of abundance within human serum) vary in their ability to bind FcγRs[10]. IgG2 and IgG4 have a reduced affinity for FcγRIIa compared to IgG1 and IgG3 and are incapable of associating with FcγRIIIb[10,11]. These differences in affinity for FcγRs likely depend on variations in the flexibility of the antibody's hinge region and specific residues within their Fc domain[10,12,13]. IgG subclass deficiencies have been described to be associated with recurrent infections, which underlines the importance of antibodies in the clearance of pathogenic bacteria from the body[14]. Furthermore, clustering of Fc receptors has been shown to be important for effective interaction with opsonized targets and thus for proper activation, intracellular signaling and phagocytosis of several cell types[15–17]. Neutrophil activation could therefore be influenced by Fc-Fc interactions and the formation of multimeric IgG.

S. aureus, a major human pathogen responsible for many community- as well as hospital-acquired infections, has developed resistance against several antibiotics (methicillin, vancomycin)[18]. Therefore, alternative treatment strategies for this pathogen are being explored and monoclonal antibody therapy is an attractive candidate[19]. Here, we observe that Fc-Fc contacts of monoclonal antibodies targeting specific staphylococcal surface



epitopes influence the direct interaction with FcγRs and subsequent phagocytosis. Using antibodies, engineered to increase Fc-Fc interactions, targeting the highly abundant staphylococcal wall teichoic acid, we show that Fc receptor-mediated phagocytosis can even be enhanced, although this effect is dependent on bacterial cell wall arrangement.
A detailed understanding of the effect of antibody binding at the bacterial surface and the influence of antibody multimerization on the interaction with phagocytes could be important for the development of highly effective monoclonal antibody therapies against pathogenic bacteria.

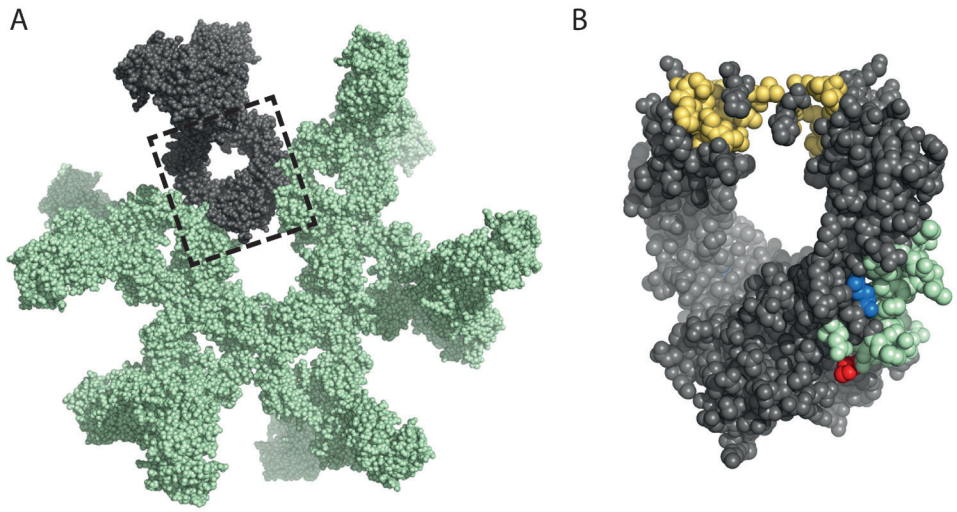
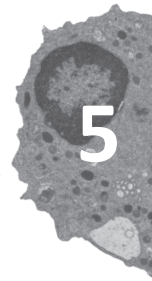


Figure 1. A. IgG hexamer crystal packing of IgG1-b12 (pdb 1HZH[39]). A single IgG is shown in grey. The dashed enclosure indicates the Fc domain. **B.** Surface map depicting the Fc domain. Residues involved in Fc-Fc interactions are shown in green[6]. Residues involved in FcγR binding are depicted in yellow[9]. Residue E430 (enhancing amino acid) is shown in blue[6,37]. Residue K439 (charge repulsion amino acid) is indicated in red[6,37].

Results

Monoclonal antibodies to specific surface antigens of Staphylococcus aureus induce Fc receptor-mediated phagocytosis

To examine the effects of the modified monoclonal antibodies (mAbs) on direct interaction with neutrophils we performed phagocytosis assays in the absence of serum and thus complement factors. First, we measured the binding of the individual wild-type (WT, lacking Fc modifications) mAbs directed against capsular polysaccharide 5 (CP5), wall teichoic acid (WTA) and clumping factor A (ClfA) to the staphylococcal surface of strain Reynolds CP5 (highly CP5 encapsulated), Newman$^{-/-}$ (CP5 encapsulated) and Wood 46 (unencapsulated) (**figure 2A**). We observed a high binding level of the CP5 antibodies to the Reynolds CP5 strain, and no binding to Newman$^{-/-}$ and Wood 46 strains. The lack of anti-CP5 binding to Newman$^{-/-}$ is due to the growth conditions of this strain which were not optimal for capsule expression. The WTA antibodies showed binding to all three strains, however the highest binding was observed for Wood 46. Binding levels of the ClfA antibodies were low for all tested strains, compared to mAbs against the other two targets. Next, we performed phagocytosis experiments with fluorescently labeled *S. aureus* in the presence of the different monoclonal WT antibodies. Fifteen minutes after addition of human neutrophils, phagocytosis was quantified by measuring the fluorescence of neutrophils by flow cytometry. The control antibody b12 did not induce phagocytosis for any of the three *S. aureus* strains. We observed that phagocytosis occurred by adding specific mAbs but that levels varied among the different antibodies (**figure 2B**). The ClfA antibodies, showing low to no measurable binding to the staphylococcal surface, were not able to induce phagocytosis on any of the strains (only shown for Newman$^{-/-}$ on which the antibody showed the highest binding). We therefore did not use this antibody for further analyses. In the presence of the CP5 antibodies, phagocytosis of the Reynolds CP5 strain was very high but no phagocytosis was observed with the other strains, which correlates with the antibody binding levels. Phagocytosis was high for all three strains in the presence of the WTA antibodies, which is in agreement with our binding assay results. These results show that Fc receptor-mediated phagocytosis can be induced by the mAbs but that it is highly dependent on the binding level of the antibody to the surface of *S. aureus*.



MAbs against capsular polysaccharide 5, modified for enhanced hexamer formation, do not show increased Fc receptor-mediated phagocytosis

Previously, we showed that modification of the Fc domain of the mAbs could change antibody multimerization and subsequent complement activation and phagocytosis (Chapter 4). Now, we assessed whether these modifications also influence IgG-Fc receptor interactions and subsequent phagocytosis via this route. Therefore, we compared the Fc-Fc contact enhancing variant E430G (**figure 1B**) to the WT antibody in phagocytosis experiments with only the mAbs as opsonins. First, we tested the monoclonal CP5 antibodies for their ability to induce phagocytosis. Therefore, we incubated fluorescently labeled Reynolds CP5 with the monoclonal CP5 antibodies and neutrophils as described before. We observed that the Fc-Fc contact enhancing variant E430G did not increase phagocytic uptake, compared

to the WT antibody (**figure 3A**). This was observed for the number of fluorescent bacteria taken up by the overall neutrophil population (**figure 3A**) as well as for the percentage of neutrophils containing one or more bacteria (**figure 3B**). To confirm the results measured by flow cytometry, we counted the bacteria per individual neutrophil of these samples by light microscopy. We observed similar results compared to the flow cytometry analyses (**figure 3C**). This shows that the modifications for enhanced Fc-Fc interactions on the mAbs targeting CP5 do not increase Fc receptor-mediated phagocytosis.

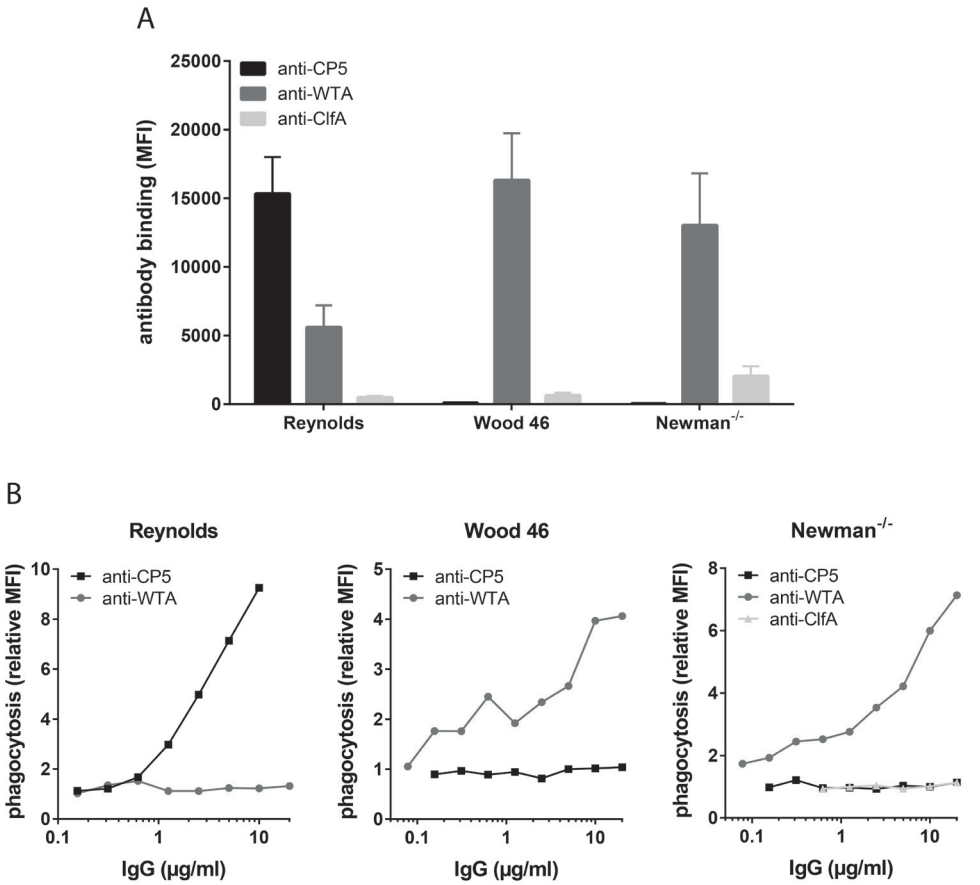


Figure 2. Monoclonal antibodies to specific surface antigens of Staphylococcus aureus induce Fc receptor-mediated phagocytosis. A. Binding of monoclonal antibodies targeting CP5, WTA or ClfA to the surface of *S. aureus* strain Reynolds CP5, Wood 46 and Newman$^{-/-}$, measured by flow cytometry. Graph represents the mean ±se of three separate experiments. **B.** Phagocytosis of GFP-labeled *S. aureus* by human neutrophils in the presence of monoclonal antibodies targeting different staphylococcal surface epitopes. This was measured by flow cytometry as the fluorescence of the neutrophils and displayed by the relative fluorescence (compared to the mean of phagocytosis in the presence of control IgG1 clone b12). Graphs are representatives of three separate experiments.

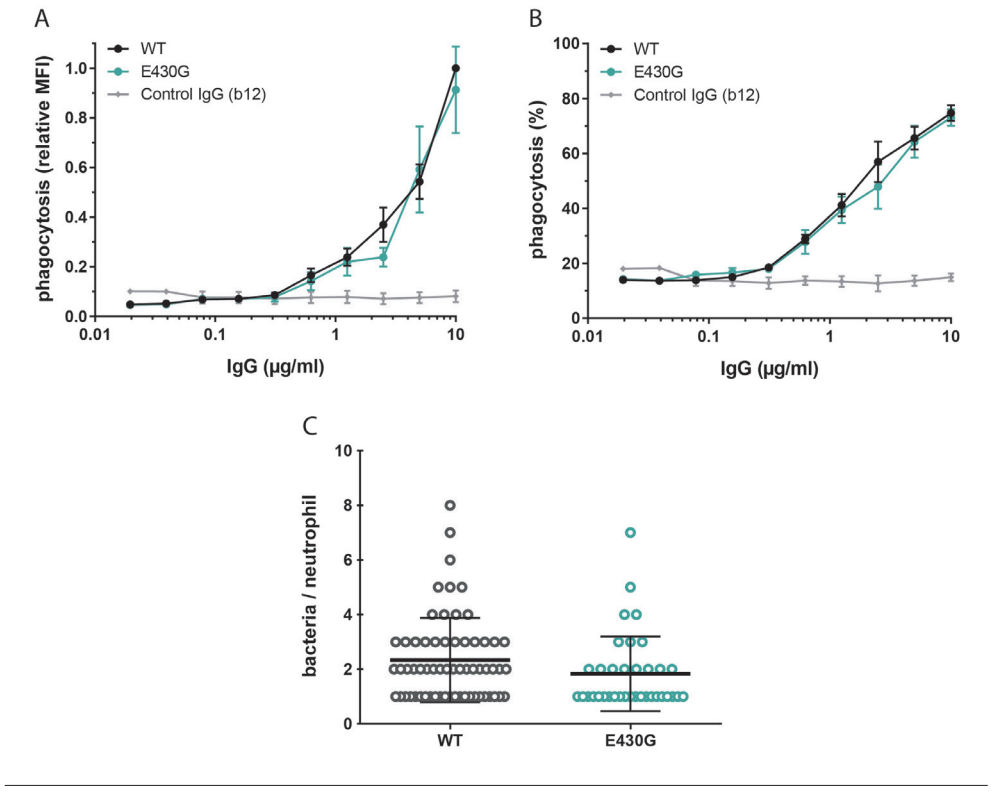


Figure 3. MAbs against capsular polysaccharide 5, modified for enhanced hexamer formation, do not show increased Fc receptor-mediated phagocytosis. A-B. Phagocytosis of GFP-labeled *S. aureus* strain Reynolds CP5 by human neutrophils in the presence of monoc1lonal WT and E430G variant antibodies targeting CP5. This was measured by flow cytometry and expressed as the fluorescence of the neutrophils and displayed by (**A**) as the relative fluorescence of neutrophils (compared to the MFL in the presence of 10 µg/ml WT mAb) or the percentage of neutrophils positive for fluorescent bacteria (**B**). Graphs represent the mean ±se of three separate experiments. **C.** Bacterial count per neutrophil of samples in the presence of 10 µg/ml mAb shown in **A-B**, as analyzed by light microscopy. Graph is a representative of three separate experiments.

MAbs against wall teichoic acid can be modified for enhanced Fc receptor-mediated phagocytic uptake

Next, we tested the monoclonal WTA antibody variants on their ability to induce Fc receptor-mediated phagocytosis. Again, we incubated *S. aureus* (Wood 46 and Newman$^{-/-}$) with the mAbs and human neutrophils and measured the fluorescence of the neutrophils by flow cytometry. Next to the WTA-WT and WTA-E430G Fc-Fc contact enhancing variant, we also tested an Fc-Fc contact inhibiting variant K439E (**figure 1B**). When phagocytosis of strain Wood 46 was measured there was a clear difference between the three antibody variants (**figure 4A**). The E430G variant induced a strong enhancement of phagocytic uptake, compared to the WT antibody. However, this effect was only observed for the number of phagocytized bacteria per neutrophil (**figure 4A**) but not for the percentage of neutrophils that had taken up one or more bacteria (**figure 4B**). Furthermore, the Fc-Fc contact inhibiting variant K439E showed a decrease in phagocytosis compared to the WT antibody (**figure 4A**).

To confirm the variation of bacterial uptake by the neutrophils we counted the bacteria per neutrophil of these samples by light microscopy (**figure 4C**). With the E430G variant, we observed a small population of neutrophils that had taken up a significantly higher number of bacteria than in the presence of the WT antibody. However, the presence of the E430G variant did not lead to an increase of bacterial uptake by the total population of neutrophils. In the presence of the K439E variant, a clear decrease of bacterial count per neutrophil was observed. Since the majority of naturally occurring antibodies against WTA are of the IgG2 subclass[20], we also tested a monoclonal IgG2 anti-WTA and its E430G variant in our phagocytosis assay (**supplemental figure 1**). This also showed an increase in phagocytosis in the presence of the E430G variant compared to the WT antibody. Although binding of both subclasses to the staphylococcal surface was similar (**supplemental figure 1A**), overall phagocytosis was significantly lower for both IgG2 variants compared to its IgG1 equivalent (**supplemental figure 1B and 1C**). This is in line with previous literature on IgG2, describing poor interaction of the immunoglobulin with Fc receptors[10]. Since binding of the WTA mAbs was also high to strain Newman$^{-/-}$ we tested this strain in phagocytosis with the antibody variants as well (**figure 4D and 4E**). The strong enhancing effect of variant E430G on phagocytic uptake of strain Wood 46 was not observed when strain Newman$^{-/-}$ was used, although a slight enhancement of phagocytosis was measured. The K439E variant, however, did show a strong reduction of phagocytosis for strain Newman$^{-/-}$ as well. Again, these effects were clearer when analyzing number of phagocytized bacteria than percentage of activated neutrophils. Overall, these findings show that Fc-Fc interactions of mAbs targeting WTA influence Fc receptor-mediated phagocytosis of *S. aureus*. This is demonstrated by a decrease in phagocytosis when Fc-Fc contact is weakened and an enhancement when stronger Fc-Fc contacts are formed, although the latter shows some diversity between strains.

MAbs against wall teichoic acid, modified for enhanced Fc-Fc interactions, can increase production of reactive oxygen species

The phagocytic uptake of opsonized bacteria leads to the production of reactive oxygen species (ROS) by the neutrophil in a process called oxidative burst[21]. We therefore measured the ROS production through a luminol-based chemiluminescence assay in samples of neutrophils incubated with *S. aureus*, opsonized with the different WTA antibody variants. We observed that at high concentrations of IgG the production of ROS in the presence of the E430G and K439E variant was comparable to the WT antibody (**figure 5A**). However, at lower IgG concentrations a clear reduction of ROS production was measured with the K439E variant compared to the WT antibody (**figure 5B, 5C and 5D**). For the E430G variant a substantial increase of ROS was observed at the lowest concentration of IgG measured compared to the WT antibody (**figure 5D**). In conclusion, decreased Fc-Fc contacts of monoclonal WTA on the staphylococcal surface will result in reduced production of ROS within neutrophils and at low IgG concentrations the enhancement of Fc-Fc interactions can significantly increase ROS production.

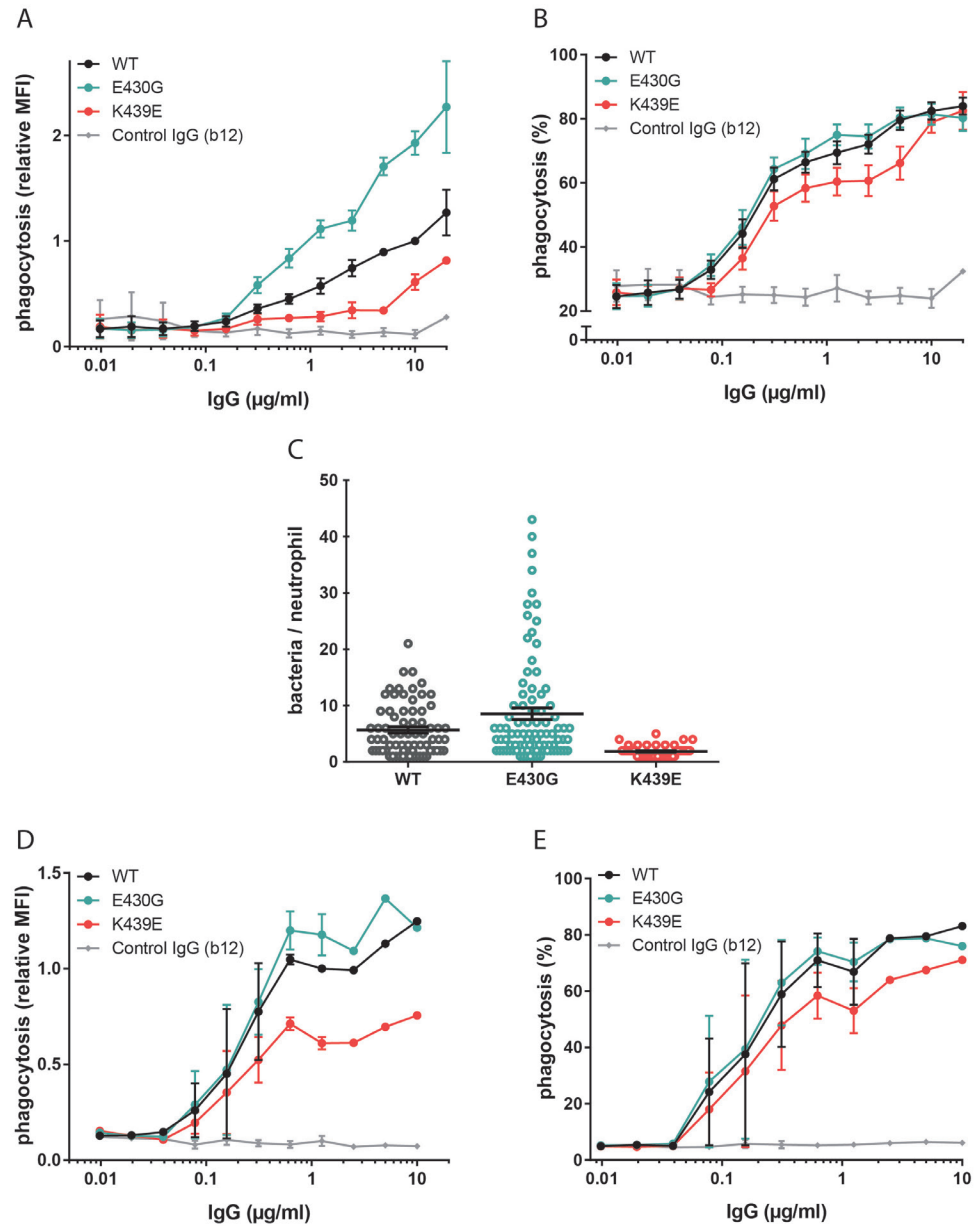


Figure 4. MAbs against wall teichoic acid can be modified for enhanced Fc receptor-mediated phagocytic uptake. A-B and **D-E.** Phagocytosis of GFP-labeled *S. aureus* strain Wood 46 (**A-B**) or Newman$^{-/-}$ (**D-E**) by human neutrophils in the presence of monoclonal WT, E430G and K439E variant antibodies targeting WTA. This was measured by flow cytometry as the fluorescence of the neutrophils and displayed by the relative fluorescence (compared to 10 µg/ml WT mAb) (**A+D**) or the percentage of neutrophils positive for fluorescent bacteria (**B+E**). Graphs represent the mean ±se of three separate experiments. **C.** Bacterial count per neutrophil of samples in the presence of 10 µg/ml mAb shown in **A-B**, as analyzed by light microscopy. Graph is a representative of three separate experiments.

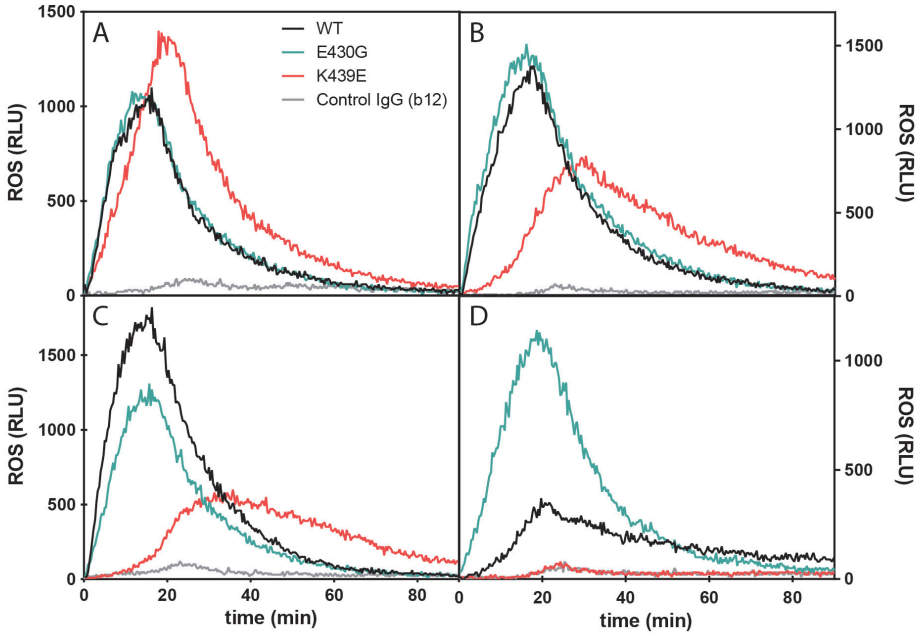


Figure 5. MAbs against wall teichoic acid, modified for enhanced Fc-Fc interactions, can increase production of reactive oxygen species. Measurement of reactive oxygen species production through luminol-based chemiluminescence assay with samples containing *S. aureus* Wood 46 opsonized with 10 µg/ml (**A**), 3 µg/ml (**B**), 1 µg/ml (**C**) or 0.3 µg/ml (**D**) monoclonal WTA antibodies and incubated with human neutrophils.

Modifications to mAbs targeting wall teichoic acid do not influence binding of soluble Fc receptors

To verify that the phagocytic uptake we are measuring in our assay is indeed mediated by Fc receptors we performed a phagocytosis experiment in the presence of FLIPr-like, a potent FcγR antagonist produced by *S. aureus*[22]. Neutrophils were pre-incubated with FLIPr-like before addition of bacteria and mAbs. Phagocytosis was enhanced in the presence of the E430G variant and decreased in the presence of the K439E variant and phagocytosis was inhibited for all mAbs by FLIPr-like (**figure 6A**). This illustrates that this process is mediated through IgG-Fc receptor interactions. Next, we assessed whether the modifications of the Fc domain of the WTA antibodies directly influenced the interaction with Fc receptors. Therefore, we incubated *S. aureus* with the WTA antibody variants and detected binding of soluble CD32a (FcγRIIa), the predominant and most potent Fc receptor on neutrophils[23]. Binding of the different WTA antibody variants to the staphylococcal surface was already shown to be comparable and this would therefore not influence the results (see chapter 4). We observed that binding of soluble CD32 to all three antibody variants was similar (**figure 6B**). This shows that the modifications to the Fc domain of the monoclonal WTA antibodies do not alter the monovalent interaction of the immunoglobulin with Fc receptors.

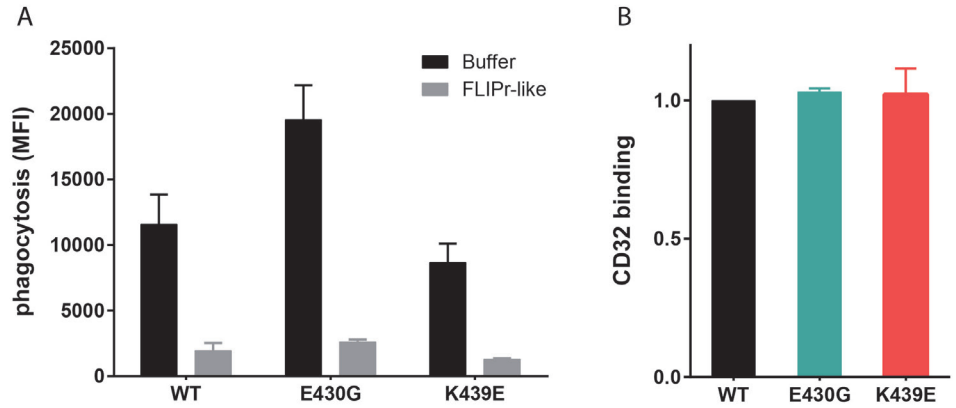
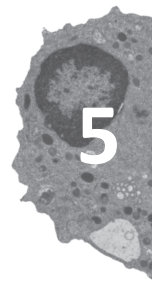


Figure 6. Modifications to mAbs targeting wall teichoic acid do not influence binding of soluble Fc receptors. A. Phagocytosis of GFP-labeled *S. aureus* strain Wood 46 by human neutrophils with 1.25 µg/ml monoclonal WT, E430G and K439E variant antibodies targeting WTA, in the presence or absence of FLIPr-like, showing the inhibitory effect of FLIPr-like on Fc receptor-mediated phagocytosis with all three antibody variants. **B.** Soluble CD32a (FcγRIIa) binding to *S. aureus* strain Wood 46, opsonized with 1 µg/ml monoclonal WT, E430G and K439E variant antibodies targeting WTA, showing comparable binding to all antibody variant (expressed relative to the WT variant). Graphs represent the mean ±se of three separate experiments.

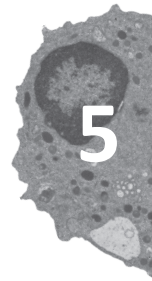


Discussion

In this study we describe the influence of Fc-Fc contacts between antibodies at the staphylococcal surface on direct interaction of these antibodies with neutrophil FcγRs. Previously, we showed that the formation of multimeric IgG is crucial for activation of the complement system via the classical pathway and for subsequent phagocytic uptake of *S. aureus* by human neutrophils (chapter 4 and [6]). Furthermore, through engineered mAbs targeting several staphylococcal surface proteins we demonstrated that complement-mediated phagocytosis can be improved by enhancing Fc-Fc interactions between IgGs. Now, we show that not only complement activation but also the direct interaction of surface-associated antibodies with neutrophil FcγRs is influenced by IgG multimerization. Using mAbs directed against WTA, modified in their Fc domain to form weakened Fc-Fc interactions, we demonstrate the necessity of multimeric IgG for efficient interaction with FcγRs and thus for subsequent Fc receptor-mediated phagocytosis. Additionally, we show that augmenting Fc-Fc interactions of WTA antibodies enhances phagocytosis. These findings are similar to the effects observed for activation of the complement system; disruption of surface-associated antibody multimers diminishes neutrophil-mediated bacterial clearance. This shows that complement- as well as Fc receptor-mediated phagocytosis is highly dependent on the formation of multimeric IgG at the bacterial surface. When performing our assays with different *S. aureus* strains we observed a small variation in binding of the monoclonal WTA antibodies to these strains, although overall binding levels were high. The anti-ClfA antibodies, however, bound significantly less to the bacterial surface as compared to the WTA antibodies. This is due to lower levels of targeted epitopes on these strains and could perhaps be improved by adjusting growth conditions to enhance expression of ClfA[24]. The ClfA antibodies were therefore not able to induce measurable levels of phagocytic uptake of *S. aureus*. The high level binding of the WT WTA antibodies resulted in efficient uptake of strain Wood 46 as well as Newman$^{-/-}$ by neutrophils. However, when multimerization of the mAbs was enhanced we did not observe an equally strong increase of phagocytosis with the Newman$^{-/-}$ strain as we did with strain Wood 46. This is likely due to the composition of the bacterial cell wall such as the density and availability of the epitopes. WTA on the bacterial membrane is substituted with either α- or β-*O*-*N*-acetyl-*D*-glucosamine (GlcNAc) and the mAbs used in our assays target β-GlcNAc[20]. The WTA on Newman could contain more repeats of β-GlcNAc than on Wood 46 and therefore bind higher densities of the monoclonal WTA antibodies[25]. If the antibodies are already in very close proximity to each other, increased multimerization would not augment binding to FcγRs. This is in line with the observed decrease in phagocytosis by the K439E variant for both strains. This variant mAb causes charge repulsion of the Fc domains and is thereby reduced in its ability to form close contacts[6]. Strain Reynolds CP5 showed moderate binding of the WTA antibodies and no measurable phagocytosis. The high density polysaccharide capsule produced by this strain has been shown to potently block phagocytosis by masking its antigens from interaction with neutrophil-associated receptors[26–28]. However, soluble molecules, such as complement factors and antibodies, were described to be able to cross the capsule[28–30]. Therefore, binding of WTA antibodies could be detected in our assays by a conjugated antibody even though

phagocytosis was completely inhibited by the capsule. The mAbs targeting CP5 were highly capable of surpassing the anti-phagocytic effect of the polysaccharide capsule. Also for these antibodies, phagocytic uptake could not be enhanced when using the E430G variant. Previously, we showed that the enhancement of Fc-Fc interactions by the monoclonal CP5 antibodies did significantly increase activation of the complement system (chapter 4). Nevertheless, interaction of the surface-associated antibodies with soluble C1 complex is incomparable to direct interaction with cell-associated receptors. Therefore, differences between these results are likely to occur. Unfortunately, we did not produce a K439E variant for the CP5 antibody and could therefore not show the effect of reducing Fc-Fc interactions on Fc receptor-mediated phagocytosis.

In conclusion, we observe that hexamerization of staphylococcal surface-associated antibodies is important for effective IgG/FcγR-mediated phagocytosis by neutrophils. Furthermore, availability and distribution of the targeted epitopes is crucial for the formation of multimeric IgG and thus for efficient neutrophil clearance. It has been suggested that both Fc receptors and complement receptors, are essential for proper activation of phagocytes[7,8]. We showed that both complement activation (Chapter 4) as well as direct FcR interaction are influenced by antibody hexamerization. Therefore, the findings described in this study are important in understanding the collective effect of antibody hexamerization at the bacterial surface on subsequent biological processes. Both aspects of antibody hexamerization should be considered in designing therapeutic strategies.



Materials & Methods

Bacterial strains and fluorescent labeling

S. aureus strains Wood 46, Newman spa^{-}/sbi^{-} (Newman$^{-/-}$) and Reynolds CP5 [28] were used in this study. Strains were fluorescently labeled with GFP by transformation with the pCM29 plasmid, constitutively expressing GFP under regulation of the sarA promoter as previously described [31,32]. The Reynolds CP5 strain was grown on Columbia agar (Oxoid) supplemented with 2% NaCl (CSA) for 24 hours at 37°C to guarantee optimal expression of the polysaccharide capsule [26,33].

Isolation of human serum and neutrophils

For preparation of serum, venous blood was collected from healthy volunteers in 9 ml vacutainer blood tubes containing a clot activator (BD). Clotting was allowed for 15 minutes and serum was collected by centrifugation at 2080g for 20 min at 4°C. Sera of 20 healthy volunteers was pooled and stored at -80°C. Heat-inactivated (HI) serum was prepared by incubation at 56°C for 30 min. For the isolation of human neutrophils, blood from a healthy donor was collected in heparin vacutainers (Greiner Bio-One) and cells were isolated using the Ficoll-Histopaque gradient method[34]. Cells were used on the same day.

Expression constructs for antibodies

For antibody expression variable heavy (VH) chain and variable light (VL) chain sequences were cloned in pcDNA3.3 expression vectors containing human IgG1 heavy chain (HC) and light chain (LC) constant regions. Desired mutations were introduced either by gene synthesis or site directed mutagenesis. Anti-MRSA Antibodies mentioned in this application have VH and VL sequences derived from previously described antibodies anti-wall teichoid acid GlcNAc beta 4497 (anti-WTA-4497; based on WO2014/193722[35]), anti-ClfA tefibazumab (based on WO2002/072600) and anti-capsular polysaccharide type 5 (anti-CP5-137G18A; based on WO2014/027698). In some of the examples the human IgG1 antibody b12, a gp120-specific antibody was used as a negative control[36]. Mutations were introduced in the Fc domain as previously described, creating antibodies modified to form enhanced hexamers (E430G mutation)[37] or weakened hexamers (S440K or K439E mutations)[6].

Transient expression

Antibodies were expressed as IgG1,κ. Plasmid DNA mixtures encoding both heavy and light chains of antibodies were transiently transfected in Expi293T cells (Life technologies, USA) using 293fectin (Life technologies) essentially as described by Vink et al.[38].

Purification and analysis of proteins

Antibodies were purified by affinity chromatography (rProtein A FF; GE Healthcare), as previously described[6]. Concentration of the antibodies was determined by absorbance at 280nm. Batches were analyzed by a number of bioanalytical assays including SDS-PAGE, size exclusion chromatography and measurement of endotoxin levels.

Antibody and CD32 binding assay

Strains Wood 46 and Newman$^{-/-}$ were cultured overnight in Todd Hewitt Broth (THB) and subcultured in fresh THB for 3h. Strain Reynolds CP5 was grown on CSA for 24h at 37°C. Bacteria were suspended in PBS, washed and resuspended in RPMI containing 0.05% human serum albumin (HSA). To determine their binding capacity, antibodies against WTA, ClfA or CP5 (all 1 µg/ml) were added and incubated for 30 min at 4°C, shaking (600 rpm). Bacteria were washed twice RPMI-HSA and incubated with either an APC-conjugated polyclonal anti-human IgG antibody ($F(ab')_2$, Jackson, 1:350) or soluble CD32a (FcγRIIa, 1 2.5 µg/ml; R&D Systems) for 45 min at 4°C, shaking (±700 rpm). For detection of CD32 binding samples were incubated with a monoclonal anti-His-tag antibody (1:100, LifeSpan BioSciences), after another wash step. Samples were washed, fixed with cold paraformaldehyde (PFA, 1%) and analyzed using flow cytometry (FACSVerse, BD).

Phagocytosis assay and bacterial count

Fluorescently labeled *S. aureus* ($1.5*10^7$/ml) was incubated with mAbs in RPMI-HSA for 15 min at 37°C. Freshly isolated human neutrophils ($1.5*10^6$/ml) were added and phagocytosis was allowed for 15 min at 37°C, shaking (750 rpm). Cold PFA (1%) in RPMI-HSA was added to stop the reaction and samples were analyzed for fluorescence of the neutrophils by flow cytometry. For the bacterial count per neutrophil, unfixed samples were centrifuged onto glass slides by cytospin, fixed and stained using Giemsa-based Diff-Quick solution and analyzed by light microscopy.

Oxidative burst assay

The luminol-enhanced chemiluminescence assay was performed in prewarmed white 96 well microplates using buffers without phenol red. *S. aureus* strain Wood 46 in IMDM (Gibco Life Technologies) plus 0.1% HSA was incubated with mAbs at 1*108/ml for 15 min at 37°C, shaking (750 rpm), in a volume of 50 µl. Neutrophils were suspended at $1.25*10^6$ c/ml in HBSS supplemented with 0.1% HSA and 150 µM luminol and 100 µl added to each well initiating the oxidative burst that was recorded continuously for 60 min at 37°C without shaking. Neutrophils incubated with only buffer served as background.

Acknowledgements

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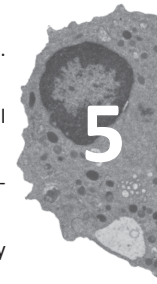
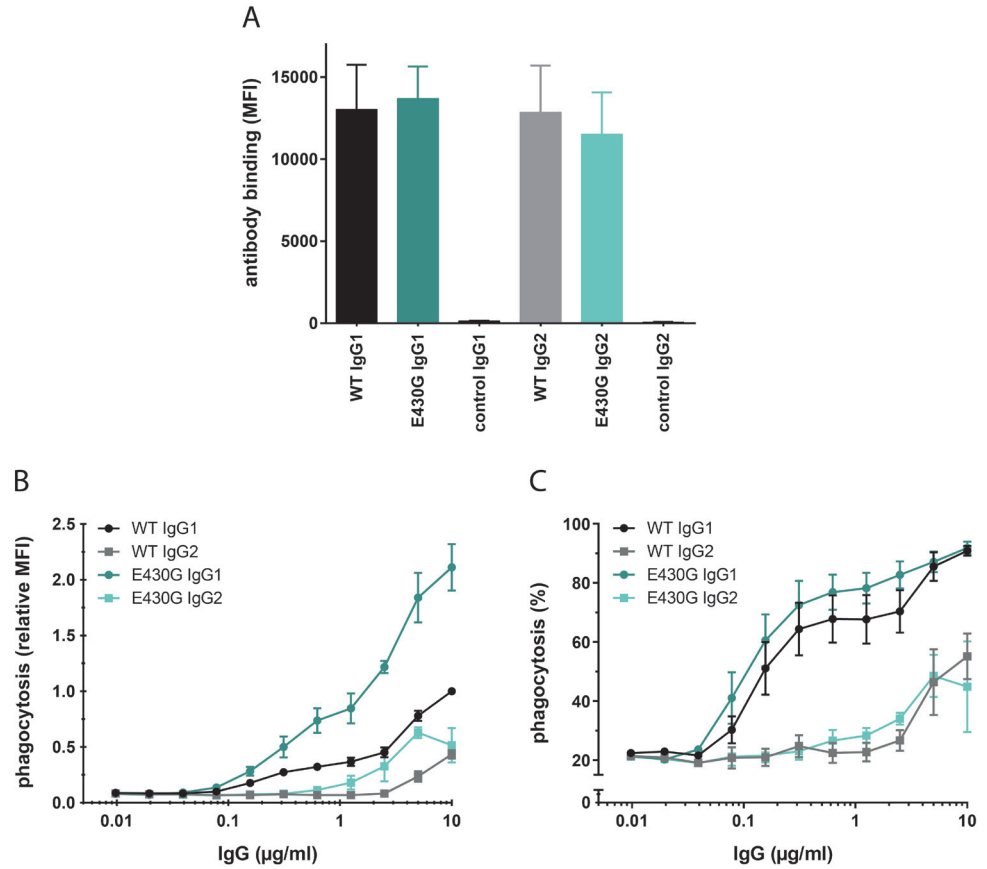
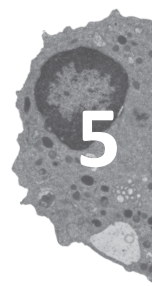


Supplemental information



Supplemental figure 1. A. Binding IgG1 and IgG2 WT and E430G variants to the surface of *S. aureus*, showing comparable binding of all variants and no binding of the control IgGs. **B-C.** Phagocytosis of GFP-labeled *S. aureus* strain Wood 46 by human neutrophils with monoclonal WT and E430G variant antibodies targeting WTA, showing enhancement of Fc receptor-mediated phagocytosis (MFI) with the E430G variant compared to WT antibody and decreased phagocytosis with the IgG2 subclass compared to the IgG1 subclass (expressed relative to 10 µg/ml of WT IgG1 variant) (**A**) or the percentage of neutrophils positive for fluorescent bacteria (**B**). Graph represents the mean ±se of three separate experiments.



|Chapter 6

Phagocyte modulation | a general discussion

Annemarie Kuipers

'Nature has provided, in the white corpuscles as you call them—in the phagocytes as we call them—a natural means of devouring and destroying all disease germs. There is at bottom only one genuinely scientific treatment for all diseases, and that is to stimulate the phagocytes. Stimulate the phagocytes. Drugs are a delusion.'
- George Bernard Shaw, 'The doctor's dilemma', 1906.

Inhibit the phagocytes

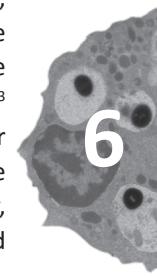
The innate immune system is the body's first line of defense against invading bacteria. Neutrophils are the innate immune system's predominant phagocytic cells and can rapidly engulf bacteria through phagocytosis, leading to intracellular killing of the microbe. Phagocytosis of bacteria by neutrophils is dependent on the direct recognition of foreign particles via specific pattern recognition receptors[1]. However, effective engulfment of bacteria highly depends on the interaction of neutrophil Fc receptors (FcRs) and complement receptors (CRs) with bacteria that are opsonized by humoral components such as antibodies and complement components. Activation of the complement cascade will lead to the deposition of the major opsonin C3b on the bacterial surface and eventually the formation of the membrane attack complex (MAC). For most Gram-negative bacteria, the formation of MAC will lead to complement mediated lysis of their membranes[2]. For some Gram-negative bacteria, additional enzymes such as serum lysozyme or amidases assist in bacterial lysis. Together this is known as serum bactericidal activity (SBA)[3]. In contrast, MAC formation does not lead to lysis of Gram-positive bacteria, such as *S. aureus*, because of their cell wall composition (discussed in chapter 1). Therefore, the main function of the complement system for this group of pathogenic bacteria is to opsonize the bacterial surface for phagocyte recognition and to produce complement cleavage products, leading to the influx of phagocytic cells. Phagocytic uptake and intracellular killing is critical for the clearance of *S. aureus* in humans. This is underlined by the fact that patients with defects in phagocyte functions, such as chronic granulomatous disease (CGD), are more prone to infections with this pathogen[4–6]. Neutrophils from CGD patients are impaired in the production of reactive oxygen species (ROS), which are crucial for the intracellular killing of phagocytized bacteria.

Phagocyte inhibition by S. aureus. To counteract these antimicrobial immune mechanisms, *S. aureus* has developed many immune-modulatory mechanisms that allow this prominent human pathogen to survive in the human body and cause a wide spectrum of diseases [7,8]. For instance, *S. aureus* has the ability to block phagocyte functions by producing proteins that either modulate binding of IgG to the bacterial surface (protein A and Sbi), inhibit recognition of surface-bound IgG by Fc receptors (FLIPr) or block activation of the complement system (e.g., SCIN, Ecb, Efb, Cna, SSL10)[8–12]. Although the staphylococcal Extracellular fibrinogen binding protein (Efb) was mainly known for its complement-inhibitory functions, in **chapter 2** we describe that this protein has potent anti-phagocytic functions. In contrast to previous studies performed with N- or C-terminal fragments of Efb, we find that the full-length (16 kDa) protein effectively blocks phagocytosis of *S. aureus* in human plasma. This is mediated by simultaneous binding to bacterium-bound C3b (via the Efb C-terminal domain) and soluble fibrinogen (via the Efb N-terminal domain).

Thereby, Efb shields bacteria with a thick layer of fibrinogen and prevents recognition of surface-associated opsonins by phagocytic cells. Indeed, we found that this Efb shield blocked the recognition of both bacterium-bound complement fragments and antibodies by neutrophils. Since this Efb-dependent fibrinogen shield seems to mediate a similar function as the *S. aureus* polysaccharide capsule, we were prompted to further study the interplay between these two shielding mechanisms. In **chapter 3** we observe that both mechanisms can coincide and perhaps even complement each other. We find that the polysaccharide capsule protects *S. aureus* from phagocytosis at low plasma concentrations but loses its protective ability at higher concentrations. Efb, however, shows a strong inhibitory effect on phagocytosis of both encapsulated as well as capsule-negative *S. aureus* strains at all tested plasma concentrations. However, no data have been produced to verify the simultaneous expression of both Efb and polysaccharides *in vivo* and therefore we cannot be certain this will occur during an infection. Expression of the two mechanism is regulated by different regulatory systems (*saeR/S* for Efb[13,14] and predominantly *agr* for capsular polysaccharides (CP)[15,16]) and for the polysaccharide capsule it was shown that this is highly variable and dependent on growth phase and the presence of environmental factors, such as CO_2 [17]. Therefore, an alternative option is that there is a spatiotemporal separation between the actions of Efb and capsular polysaccharides during infection. Both expression of Efb and CP have been shown to contribute to *S. aureus* virulence in mice and are thus important for its survival. Additionally, *S. aureus* has been shown to induce abscess formation whereby the bacterium shield itself from the surroundings by formation of capsule-like structures[18]. Efb has previously been described to be involved in the formation of abscesses[19]. Also purified CP8 and CP5 have been shown to provoke abscess formation[20]. Therefore, both capsule and the Efb-fibrinogen shield could be implicated in abscess formation and thereby contribute to *S. aureus* virulence. Furthermore, the presence of a thick shield, existing of fibrinogen, might induce high expression of staphylococcal proteins regulated by *agr*, since this system is upregulated when sensing high cell densities[21] and in this case confinement by a fibrinogen shield. For the same reason, the function of antibiotics could be impeded by a dense fibrinogen capsule covering the bacterium. However, these hypotheses have not yet been confirmed by experimental studies.

Since encapsulation of the bacterium, by polysaccharides as well as by the Efb-induced fibrinogen shield, will hide the bacterial surface from its surroundings, the inhibition by these mechanisms is not specific for a distinctive human cell receptor or even cell type. Therefore, next to neutrophil phagocytosis, also recognition and clearance by other important immune cells, such as macrophages and dendritic cells and thus further presentation to the adaptive immune system will be prevented as well. Since *S. aureus* is a major cause of bacteremia[22,23] which will result in the accumulation of the bacteria in the liver, the escape from Kupffer cells, the predominant phagocytic cells at this location[24], will be crucial for the survival of the bacterium and should hypothetically also be established by these mechanisms. In this way, these mechanisms are quite similar and have broader immune evasive properties compared to the specific functions of other immune evasion proteins secreted by *S. aureus*.

Modulation of coagulation. To prevent clotting, our experiments with Efb were performed in the presence of plasma that was treated with a direct thrombin inhibitor. Therefore,



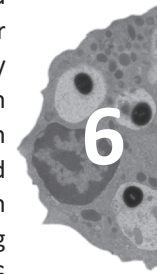
conversion of fibrinogen to fibrin was not possible and our findings do not allow fibrin formation at the bacterial surface after Efb binding. The formation of fibrin fibrils is an important antimicrobial mechanism by the host since it will entrap bacteria within a fibrin network[25]. However, *S. aureus*, and other pathogens, have developed methods to use the formation of fibrin to their advantage. For instance, the secreted *S. aureus* coagulases (Coagulase and von Willebrand factor binding protein) have the ability to bind prothrombin, which induces a conformational change that leads to its non-proteolytic activation into thrombin. In this way, coagulases induce the conversion of fibrinogen to fibrin and the formation of a clot and they have been found to be important virulence factors[26–28]. The fibrin networks created by *S. aureus* are suggested to be structurally different from conventional clots and are thereby beneficial for escaping phagocytic clearance in the tissues and will promote bacterial growth and dissemination through the blood[26,29]. Efb may be able to aid coagulases in that it can bind fibrinogen to the bacterial surface for formation of fibrin in close proximity to the bacterium, just as was recently suggested for Coagulase itself[30]. Furthermore, *S. aureus* expresses fibrinogen-binding surface proteins (ClfA, FnbpA[31,32]) that promote aggregation of the bacteria but also of platelets in the bloodstream[33,34]. Next to the misuse of fibrinogen by the pathogen, *S. aureus* also secretes molecules that can activate other coagulation factors. For instance, activation of the contact pathway of coagulation is induced by *S. aureus* staphopains (ScpA and SspB) to release bradykinin (BK) from high molecular weight kininogen (HK), resulting in enhanced vasodilation and the influx of plasma nutrients and dissemination of the bacteria into the bloodstream[35,36]. However, the secretion of several molecules by *S. aureus* and other pathogens that promote fibrinolysis (e.g. *S. aureus* staphylokinase (SAK), streptococcal streptokinase (STK), *Yersinia pestis* Pla[37,38]), indicates that the inhibition of coagulation functions is also important for its proliferation within the host. This again suggests that the different locations within the host require alternative mechanisms of action for the survival of *S. aureus*.

Stimulate the phagocytes

Since phagocytosis is crucial for the clearance of *S. aureus* from the body, one way to promote clearance of this devastating bacterium is to improve the activity of our immune system. Therapeutic antibodies offer one way to enhance opsonization and subsequent killing of *S. aureus* by phagocytes. However, the development of such alternative therapies is severely hampered by the lack of insights into antibody-dependent complement activation. In **chapter 4** we show for the first time that antibodies against pathogenic *S. aureus* require organization into hexameric structures to induce complement activation and phagocytosis by neutrophils [patent pending]. In a recent Science paper it was shown that antibodies effective in complement activation bind to targets on surfaces in a highly ordered manner: IgG antibodies form hexamers on the cell surface following antigen binding[39]. This hexamerization of antibodies is critical for optimal C1q binding, complement activation and complement-mediated killing. In addition, Genmab identified mutations that enhanced IgG clustering after binding to cells which led to an increase in C1q binding and complement dependent cytotoxicity (CDC). Such a promising approach for increasing the efficacy of therapeutic antibodies may improve antibody therapy for infections.

Antibody hexamerization. Our data in **chapter 4** indicate that IgG molecules, present in human immune sera, require Fc-Fc contacts to mediate effective opsonization of the bacterium with complement C3b and subsequent phagocytosis. This was demonstrated using an Fc-binding peptide that interacts with the region for Fc-Fc interactions in IgG hexamers[39]. Incubation with the Fc-binding peptide had already been shown to interfere with the Fc-Fc interactions by monoclonal IgG, resulting in decreased complement activation and CDC on tumor cells[39]. In our study we show that also in normal human serum, consisting of polyclonal antibodies targeting *S. aureus*, addition of the Fc-binding peptide potently blocked complement deposition and, importantly, subsequent phagocytic uptake by neutrophils. Furthermore, monoclonal antibodies engineered to generate enhanced hexamer formation showed significantly increased complement activity on and phagocytic uptake of *S. aureus*. Interestingly, our data with monoclonal antibodies indicate that the density of antigens is crucial for the arrangement of IgG into hexamers and thus the effective initiation of the complement system. This was suggested from the functional comparison between human IgG1 molecules against Clumping factor A (ClfA), a cell-wall anchored surface protein, and Wall teichoic acid (WTA), a highly abundant glycopolymer comprising 40% of the bacterial cell wall. While antibodies against WTA could be mutated for enhanced complement activation, this could not be demonstrated for ClfA antibodies. These data seem in contrast to previous data on tumor cells, where enhanced IgG hexamerization could be demonstrated for relatively low-density epitopes (lower than WTA)[39]. However, a crucial difference might be that protein epitopes on tumor cells are present in a fluidic membrane where epitopes can move[40]. Especially on Gram-positive bacteria, antibody epitopes are linked to a rigid cell wall (peptidoglycan) and have less flexibility than membrane proteins[41]. With this in mind, the effects measured with the WTA and CP5 antibodies could potentially be further improved when multiple bacterial epitopes are targeted simultaneously, thus increasing the chance of IgG molecules to interact. It has been shown that antibodies do not remain stationary on surfaces of both bacteria and viruses but can dissociate one of their Fab fragments from the targeted epitope to relocate to another nearby epitope, thereby inducing the clustering required for complement activation and phagocytosis[42]. Also, for arrangement of the antibodies into hexamers the release of one of the Fab domain from the bacterial surface is required[39] (**figure 1**). Therefore, the affinity of the antibody for the epitope it will target can also not be very high, since release will then be impeded.

Although hexameric IgG molecules provide an optimal docking for C1q, it has been reported that lower order multimers of IgG can also be formed at the cell surface[39]. Therefore, our observations on the importance of IgG multimerization on *S. aureus*, do not necessarily imply that IgG hexamers are formed. The technical limitations to study hexamer formation are thus an interesting challenge for future research. Modifications made to the Fc domain of the monoclonal antibodies for enhanced and weakened hexamer formation are based on a crystal structure of an anti-HIV molecule arranged into a hexamer[39]. The visualization of C1q bound to an IgG hexamer were previously studied on the surface of liposomes using cryo-EM[39]. Future studies should be performed to address the exact arrangement of IgG molecules on *S. aureus*. Potentially, recent developments in electron cryotomography on bacterial cells can contribute to studying antibody arrangements on bacteria[39,43,44]. In addition, it would be favorable to analyze hexamerization on the bacterial surface in a high



throughput manner.

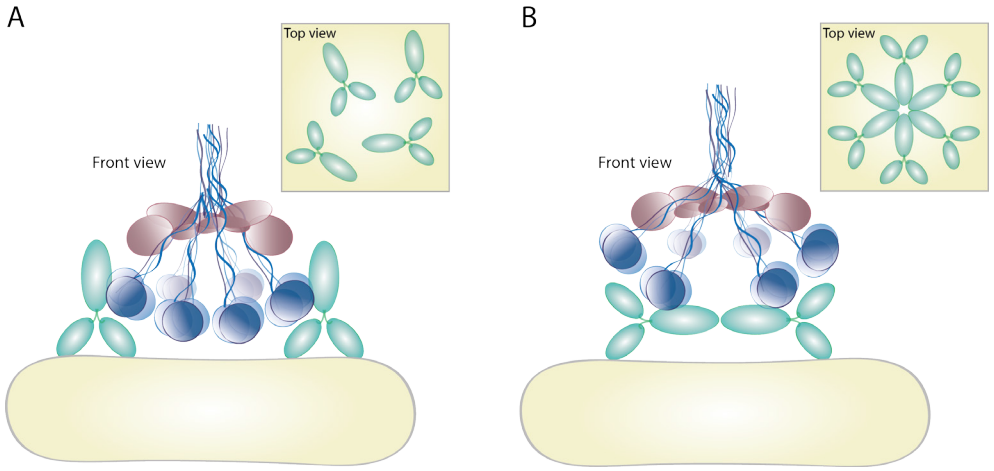


Figure 1. Schematic representation of the C1 complex (in blue/taupe) bound to antibodies (in green), on to the bacterial surface (in yellow). **A** represents the classical view of randomly distributed antibodies bound the bacterial surface with both Fab domains and C1 binding to antibodies in close proximity. **B** represents the theory by Diebolder *et al* and our studies of highly arranged hexamers by Fc-Fc interactions requiring the release of one of the Fab domains from the bacterial surface, resulting in strong C1 binding and high complement activation.

Interaction with Fc receptors. Phagocytosis of *S. aureus* by neutrophils and the enhancing effect of increased hexamer formation could be a result of several processes. The opsonization of the bacteria with complement, particularly C3b, will lead to recognition by complement receptors on the neutrophil. However, surface-bound antibodies will be recognized directly by Fc gamma receptors (FcγRs) as well and this process will therefore also be involved when measuring phagocytosis in normal human serum. Therefore, in **chapter 5** we focused on the effects of altering hexamer formation on the direct interaction with FcγRs. We find that also FcγR-antibody interaction is influenced by the Fc-Fc interactions of IgG. This influence was more prominent when disrupting Fc-Fc interactions than when this arrangement of IgG was enhanced. Again, this effect was highly dependent on cell wall composition and thus availability and density of the targeted epitopes. Furthermore, the subclass of IgG impacts phagocytosis and the additional effect of enhanced hexamerization as well, showing lower uptake in the presence of IgG2 for both the WT antibody and the variant inducing enhanced hexamerization, compared to the IgG1 equivalents. Once more this shows how the above-described criteria will affect the results of these studies.

Implications for other bacteria. Although the studies in this thesis show the importance of IgG multimerization on *S. aureus*, we anticipate that phagocytosis by other Gram-positive bacteria is also mediated by IgG hexamers. Potentially, these insights are also relevant for human immune protection against Gram-negative bacteria. The main effector of complement on Gram-negatives is the ring-structured pore-forming complex, MAC, that disintegrates bacterial membranes[45,46] Antibody therapy against Gram-negative bacteria is mainly focused at developing antibodies that elicit potent killing by the MAC[3]. However we

currently do not understand why certain antibodies mediate bacterial killing while others do not. Potentially the multimeric arrangement of antibodies described in this thesis, also play a role in antibodies mediating SBA.

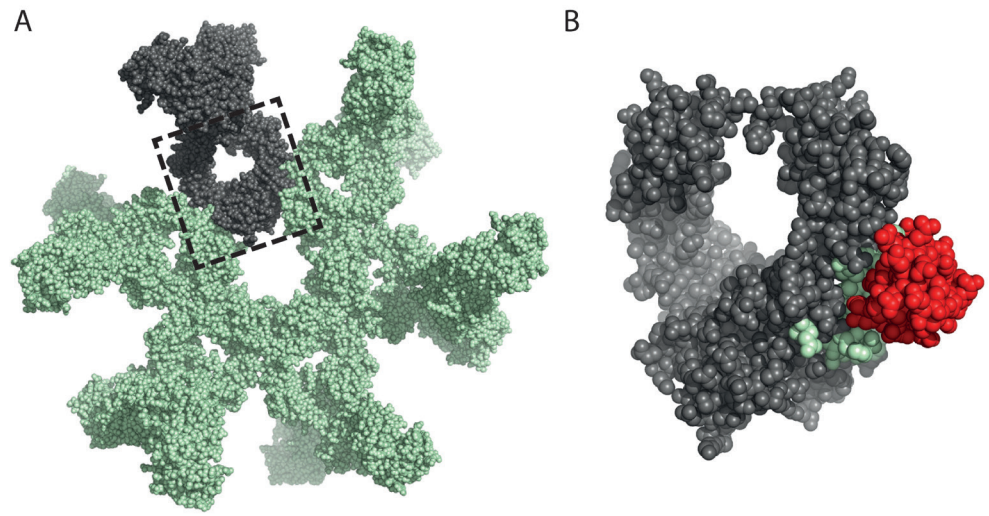


Figure 2. A. IgG hexamer crystal packing of IgG1-b12 (pdb 1HZH[52]). A single IgG is shown in grey. The dashed enclosure indicates the Fc domain. **B.** Surface map depicting the Fc domain. Residues involved in Fc-Fc interactions are shown in green[39]. Interaction of staphylococcal protein A is shown in red (pdb 1FC2: Fc domain docked to IgG1-b12 Fc domain, 1FC2 Fc domain hidden).

Drugs are a delusion?

The antibiotic resistance of *S. aureus* (MRSA, VRSA) but also many other bacteria (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, ESBL producing *E. coli*) forms a vast threat for public health and therefore novel treatment strategies are needed to control these infections[47]. Improving the activity of our immune system, or 'stimulating the phagocytes', through therapeutic antibodies is one approach[48–50]. Antibody therapy is already well established in cancer treatment but not yet in infectious diseases[51]. The development of monoclonal antibodies in infectious disease will be a fast and convenient alternative next to the existing approaches, also since it is specific and has limited side effects. Concerning *S. aureus*, antibody therapy would be very interesting in clinical settings as a prophylactic during surgery to prevent wound infections.

Only one commercially available monoclonal antibody was produced for infectious diseases (RSV, Paluvizumab) during the last decade, although many companies are now testing monoclonal antibacterial antibodies in phase I, II and III studies (anti-*E. coli,* anti-*Klebsiella,* anti-*S. aureus*, anti *P. aeruginosa*). Therefore, detailed basic understanding of what a good opsonic antibody for bacteria should entail is key for the production of these treatment approaches. Since we now showed, for the first time, that antibodies against *S. aureus* organize highly arranged structures through Fc-Fc interactions to induce enhanced complement activation and subsequent clearance by neutrophils, this illustrates an interesting approach

for improving the efficacy of therapeutic antibodies against bacterial infections in general. However, staphylococcal evasion mechanisms such as the formation of an Efb-fibrinogen shield and the expression of surface proteins protein A and Sbi, which bind the Fc domain of antibodies and thereby block their opsonic properties (**figure 2**), could hinder the binding or correct binding of opsonic antibodies. Therefore, enhancing multimeric arrangement at the bacterial surface would not be established or increase phagocytic clearance. The production of an effective therapy could thus require combining both neutralizing antibodies that can block functions of secreted proteins (e.g. Efb) or surface proteins (e.g. protein A), as well as opsonizing antibodies targeting exposed surface structures, such as WTA and CP.

In conclusion, our studies indicate that the mechanisms developed by *S. aureus* to **inhibit the phagocytes** could be overcome by new mechanisms to **stimulate the phagocytes** with the right antibodies.

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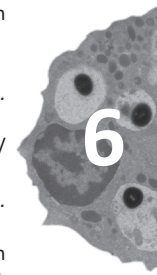

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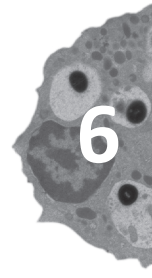

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Closing pages

Nederlandse samenvatting

Zo kort mogelijk

Het aangeboren afweersysteem is in staat om veel van de binnengedrongen ziekteverwekkers, zoals bijvoorbeeld bacteriën, uit het lichaam te verwijderen. Hiervoor zijn witte bloedcellen en vooral de neutrofielen erg belangrijk, aangezien zij de bacteriën kunnen opnemen (fagocyteren) en onschadelijk kunnen maken. Deze neutrofielen worden geactiveerd en naar de bacteriën toegetrokken door verschillende moleculen in het menselijke bloed. Essentieel hiervoor zijn antilichamen en het complementsysteem, bestaande uit eiwitten die worden geactiveerd door het signaleren van bacteriën. Als gevolg hiervan worden sommige van deze complementfactoren afgezet op het bacteriële oppervlak wat leidt tot herkenning van de bacterie door neutrofielen, resulterend in fagocytose. *Staphylococcus aureus* (*S. aureus*) is een bacterie die normaal gesproken op de huid en achterin de neus voorkomt en daar geen ziekte veroorzaakt. Helaas komt het regelmatig voor dat deze bacterie binnendringt via wondjes in de huid en zo wel ernstige infecties veroorzaakt. De bacterie is bekend vanwege zijn steeds meer voorkomende resistentie tegen de meest gebruikte antibiotica (MRSA) en de veel voorkomende infecties in de ziekenhuisomgeving. De mogelijkheid van *S. aureus* om ziektes te veroorzaken is grotendeels te danken aan de productie van moleculen die in staat zijn belangrijke processen van de aangeboren afweer af te remmen of volledig te blokkeren. Eén van de mechanismen om afweerreacties te remmen is de productie van het eiwit Efb (Extracellulair fibrinogeen-bindend eiwit) en wordt beschreven in dit proefschrift. Dit uitgescheiden eiwit is in staat sommige van de complementfactoren, die aan het bacteriële oppervlak bevestigd zijn, te binden en op het zelfde moment het humane eiwit fibrinogeen uit het plasma naar het oppervlak toe te trekken. Hiermee vormt het een schild om de bacterie waarmee het bacteriële oppervlak afgeschermd wordt van herkenning door neutrofielen. Dit resulteert in de remming van fagocytose en verhoogt daarmee de overleving van de bacterie in het menselijk lichaam. Aan de andere kant is het immuunsysteem zeer goed in staat verschillende onderdelen van bacteriën te herkennen. Helaas zijn ernstige infecties met *S. aureus* niet ongewoon en wordt er daardoor veel onderzoek gedaan naar mechanismen om deze immuunprocessen te versterken en daarmee infecties te voorkomen of te verminderen. Onlangs werd ontdekt dat antilichamen, die gebonden zijn aan het oppervlak van tumorcellen, interactie met elkaar kunnen aangaan door middel van hun 'staart' (Fc-domein) en hierdoor een structuur van zes aan elkaar gebonden antilichamen, een hexameer, kunnen vormen. Deze hexameren zijn beter in staat complement te activeren dan een enkel antilichaam. Wij tonen hier aan dat ook voor het activeren van complement op het oppervlak van bacteriën deze formatie van hexameren van antilichamen erg belangrijk is. Wanneer de hexameren worden verbroken is er nauwelijks complementactiviteit aanwezig en worden de bacteriën dus niet herkend en gefagocyteerd. We laten zien dat, door middel van antilichamen die aangepast zijn in hun Fc-domein, we het vormen van hexameren van antilichamen kunnen verhogen en hiermee de complementactiviteit en opvolgende fagocytose van *S. aureus* kunnen versterken. Deze bevindingen zouden gebruikt kunnen worden om antilichaamtherapieën te verbeteren die worden ontwikkeld voor het behandelen van *S. aureus* infecties.

Introductie

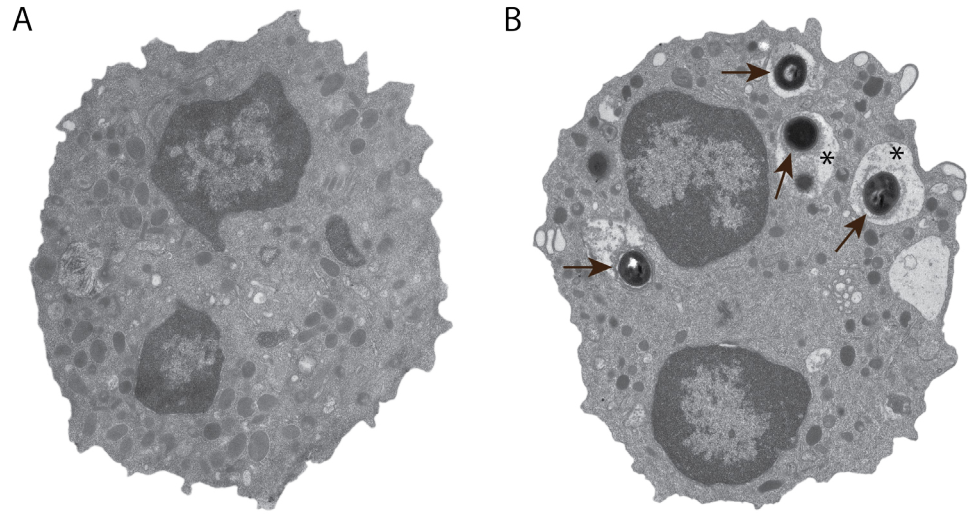
Het menselijk aangeboren immuunsysteem is erg belangrijk als de eerste afweer tegen ziekteverwekkers wanneer zij het lichaam zijn binnengedrongen. Dit systeem werkt nauw samen met het adaptieve immuunsysteem, dat specifieker kan reageren op deze ziekteverwekkers. Een onderdeel van de adaptieve immuniteit zijn de antilichamen. Deze eiwitten worden geproduceerd door witte bloedcellen (genaamd B-cellen) tegen specifieke structuren (antigenen of epitopen) op zowel ziekteverwekkers als veranderde eigen cellen, zoals tumorcellen. Hoewel ze worden geproduceerd door cellen van het adaptieve immuunsysteem, zijn ze ook erg belangrijk voor de aangeboren immuunreactie. Antilichamen (ofwel immunoglobulinen (Ig)) zijn Y-vormige eiwitten waarvan de twee armen, de Fab-domeinen (Fab: Fragment antibody binding), kunnen variëren en zo verscheidene epitopen kunnen herkennen. De staart wordt het Fc-domein (Fc: Fragment crytallizable) genoemd. De Ig's kunnen onderverdeeld worden in verschillende klassen (IgA, IgD, IgE, IgG, IgM), gebaseerd op het type Fc-domein. Deze klassen worden geproduceerd voor specifieke doeleinden. IgG is het meest voorkomende antilichaam in het bloed en is zeer belangrijk voor de afweer tegen bacteriën.

Minder bekend, maar ook cruciaal voor de menselijke aangeboren afweer is het complementsysteem. Dit systeem bestaat uit zo'n dertig eiwitten die in het bloed circuleren en geactiveerd worden na herkenning van specifieke structuren (op bijvoorbeeld bacteriën) of door het signaleren van aan het oppervlak gebonden antilichamen. Deze herkenning leidt tot een cascade (kettingreactie) van omzettingen van de andere moleculen van het complementsysteem. Het systeem kan op verschillende wijzen worden geïnitieerd waardoor drie verschillende routes geactiveerd worden: de klassieke route, de lectine route of de alternatieve route. De drie routes resulteren uiteindelijk in dezelfde eindproducten. De klassieke complementroute (die centraal staat in dit proefschrift) wordt geactiveerd wanneer het complementcomplex C1 antilichamen herkent die zijn gebonden aan het oppervlak van bijvoorbeeld tumorcellen of bacteriën. C1 bevat zes antilichaam-bindende 'koppen' waarvan is aangetoond dat ze meerdere antilichamen moeten binden om zo'n sterke interactie aan te gaan dat deze zorgt voor activatie van het complex. Deze activatie leidt tot de formatie van convertases: oppervlak-gebonden complexen die complementfactoren kunnen knippen en deze daarmee kunnen activeren. De convertases kunnen complementfactor C3 omzetten in C3a en C3b. Deze laatste component, C3b, wordt afgezet op het oppervlak van bijvoorbeeld de bacterie en is essentieel voor het markeren, ofwel opsoniseren (van het Griekse *opsonin*: voorbereiden op het eten), van de bacterie voor herkenning door immuuncellen. Het omzetten van C3, in onder andere C3b, is waar de drie complementroutes samenkomen. Daarnaast vormt dit C3b weer nieuwe convertases die complementfactor C5 om kunnen zetten en daarbij C5a kunnen afsplitsen. Het C5a is zeer belangrijk voor chemotaxie, het aantrekken van immuuncellen naar de plek van een ontsteking. Het uiteindelijke product van de complementroutes is de formatie van het MAC (Membrane Attack Complex). Dit complex bestaat uit component C5b-9 en is in staat een ringstructuur te vormen op bijvoorbeeld tumorcellen en sommige bacteriën. Met deze ring kan het complex de membraan verbreken en zo de cellen doden. Zo kan activatie van het complement-systeem direct leiden tot het opruimen van bacteriën en tumorcellen.

Naast het humorale (zich bevindend in de vloeistof, dus het serum/plasma) deel van het bloed zijn ook de witte bloedcellen cruciaal voor de afweer tegen ziekteverwekkers. Erg belangrijk voor de acute, eerste fase van een infectie zijn specifieke witte bloedcellen genaamd neutrofiele granulocyten of kort, neutrofielen. Deze cellen zijn in grote getalen aanwezig in het bloed en zullen als eerst reageren door hun vermogen om snel vanuit de bloedbaan te migreren naar de plek van infectie. Ze worden hiertoe gestimuleerd door moleculen genaamd chemoattractanten (stoffen die tot chemotaxie aanzetten), zoals C5a maar ook kleine moleculen die door de bacterie zelf worden uitgescheiden. Nadat de neutrofielen naar de plek van infectie zijn gemigreerd, herkennen ze ziekteverwekkers door middel van receptoren op hun oppervlak. Er bevinden zich vele receptoren op de neutrofiel, gericht tegen een variëteit aan liganden (moleculen die specifiek voor die receptor zijn). Er zijn onder andere specifieke complementreceptoren en receptoren die direct oppervlak-gebonden antilichamen kunnen herkennen (Fc-receptoren). Na herkenning van de schadelijke of infecterende bron door receptoren zal de neutrofiel aangezet worden tot het opnemen van de bacterie of cel (fagocyteren (**figuur 1**), van het Griekse *phagein*: eten/verslinden). Hiermee behoort de neutrofiel tot de groep cellen die professionele fagocyten genoemd worden. Nadat de ziekteverwekker of cel is opgenomen in een zogenaamd fagosoom, zal deze onschadelijk worden gemaakt door fusie van dit fagosoom met andere compartimenten in de cel. Deze compartimenten bevatten verschillende enzymen die de ziekteverwekker of beschadigde cel kunnen afbreken.

Ondanks ons zeer uitgebreide en complexe immuunsysteem zijn er toch bacteriën die kunnen overleven in het menselijk lichaam en daar ziektes veroorzaken, de zogenaamde pathogene bacteriën. In ons lichaam bevinden zich ook duizenden verschillende soorten commensale bacteriën. Deze richten daar geen schade aan en zijn zelfs belangrijk voor verschillende processen, zoals het verteren van voedsel. De van nature commensale bacterie *Staphylococcus aureus* (*S. aureus*) leeft bij meer dan een derde van de mensen achterin de neus en op de huid, zonder enigszins schadelijk te zijn. Wanneer de huid beschadigd raakt kan deze bacterie echter het lichaam binnendringen en ernstige infecties veroorzaken zoals wondinfecties, longinfecties, meningitis en sepsis. Daarnaast heeft de bacterie resistenties ontwikkeld tegen veel gebruikte antibiotica, zoals meticilline (MRSA), en is hij vaak de veroorzaker van ziekenhuisinfecties. Tot nu toe is het niet gelukt een effectief vaccin te ontwikkelen tegen de bacterie. Hierdoor is men op zoek naar geschikte alternatieven voor de behandeling van *S. aureus* infecties, zoals antilichamen gericht tegen de bacterie die de opname door fagocyten kunnen verhogen.

Eén van de redenen dat *S. aureus* zo goed is in het overleven in het menselijk lichaam, is het vermogen om tal van eiwitten uit te scheiden die diverse processen van het immuunsysteem kunnen remmen of volledig blokkeren. Zo produceert *S. aureus* eiwitten die de productie van opsoninen of de binding hiervan aan het bacteriële oppervlak kunnen remmen en daarmee ook fagocytose kunnen belemmeren. Dit maakt de bacterie lastig te verwijderen uit het menselijk lichaam en hindert het produceren van een effectief *S. aureus* vaccin.

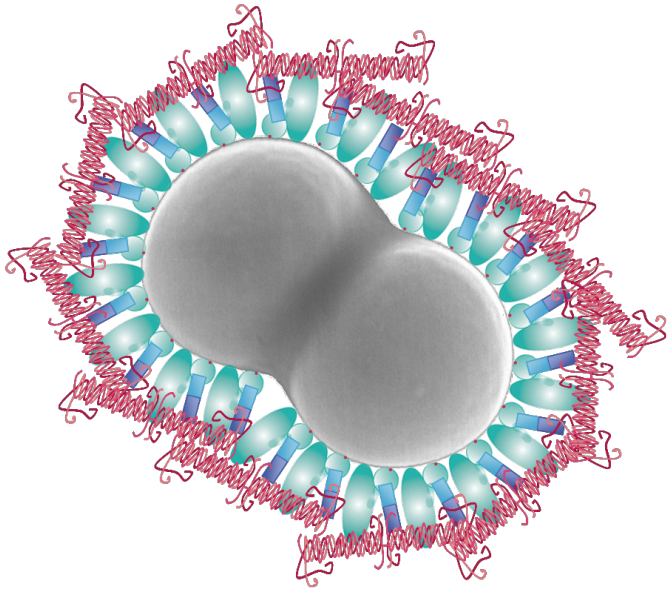


Figuur 1. Elektronenmicroscopie-afbeelding van een neutrofiel vóór (**A**) en na (**B**) fagocytose van *S. aureus*. De pijlen geven gefagocyteerde bacteriën aan. De fagosomen zijn aangegeven met een asterisk.

Dit proefschrift

In dit proefschrift wordt het onderzoek beschreven naar zowel een mechanisme van *S. aureus* voor het remmen van fagocytose als naar een mechanisme om de opname van deze bacterie door neutrofielen juist te verhogen.

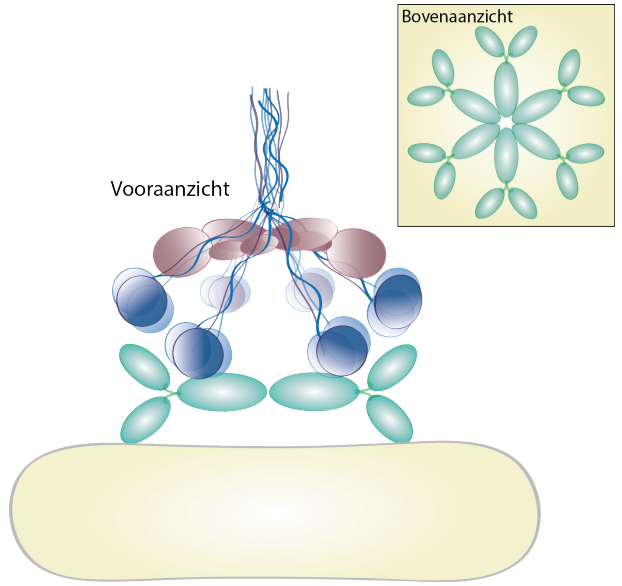
S. aureus heeft meerdere mechanismen ontwikkeld om onderdelen van het menselijke immuunsysteem te ontwijken, waaronder ook het complementsysteem. Eén van deze eiwitten is het Extracellulair fibrinogeen-bindend eiwit (Efb). Van dit kleine (16 kDa), uitgescheiden eiwit was al bekend dat het begin van het eiwit (de N-terminus) kan binden aan het menselijke plasma-eiwit fibrinogeen, dat belangrijk is voor de vorming van bloedstolsels bij de wondheling. Daarnaast was al aangetoond dat het eind van het eiwit (de C-terminus) kan binden aan C3b en convertases die deze component bevatten. Daarmee verhindert het de omzetting van de verdere cascade van het complementsysteem. Niet bekend was waarom beide functies zich op hetzelfde eiwit bevinden. In **hoofdstuk 2** laten we zien dat Efb zich bindt aan het C3b dat afgezet is op het bacteriële oppervlak en daarnaast fibrinogeen naar het oppervlak kan trekken (**figuur 2**). Hiermee vormt het een schild van fibrinogeen om de bacterie waarmee het oppervlak (en dus alle epitopen) maar ook gebonden antilichamen en complementfactoren afgeschermd worden van herkenning door fagocyten, zoals neutrofielen. Dit dikke schild kunnen we ook goed zichtbaar maken met microscopie. We hebben hiermee aangetoond dat *S. aureus* met het uitscheiden van Efb zeer effectief fagocytose kan remmen en dus de overleving in het menselijk lichaam kan verbeteren.



Figuur 2. Schematische voorstelling van het mechanisme van Efb, met in het grijs *S. aureus*, in het groen C3b, in het blauw Efb en in het rood fibrinogeen.

Ongeveer 75% van alle klinische *S. aureus* stammen produceert een kapsel van suikers (polysachariden). Van bepaalde stammen die een dik kapsel produceren is bewezen dat dit, net als het schild dat Efb vormt, het oppervlak afschermt van herkenning door het menselijk immuunsysteem. In **hoofdstuk 3** onderzochten we of stammen die dit polysacharidekapsel vormen nog toegankelijk C3b op het oppervlak hebben en zo ook een Efb-schild kunnen vormen. Allereerst laten we zien dat vooral de bekende Reynolds stam, die zeer veel kapsel produceert, hiermee opname door fagocyten kan remmen. De Reynolds stam produceert één van de meest voorkomende typen *S. aureus* kapselpolysachariden, genaamd CP5. Een ander veel voorkomend kapseltype is CP8. We hebben verscheidene CP5- en CP8-producerende *S. aureus* stammen getest. Deze stammen konden nauwelijks fagocytose remmen, vergeleken met niet kapsel-producerende stammen of dezelfde stammen die gemodificeerd waren zodat zij geen kapsel meer produceerden. Voor de Reynolds stam laten we zien dat vooral in lage concentraties plasma (die bijvoorbeeld in weefsel voor zouden kunnen komen) fagocytose geremd wordt. In hogere concentraties plasma (bijvoorbeeld bij een influx van fagocyten en dus ook plasma vanuit het bloed in het weefsel tijdens een ontsteking) heeft het kapsel geen remmend effect meer op opname door fagocyten. Wanneer Efb aanwezig is, zien we dat voor alle stammen, zowel kapsel-vormend als kapsel-negatief, fagocytose sterk geremd kan worden. Dit effect is zichtbaar in zowel lage als hogere concentraties plasma, waar het kapsel geen afschermend effect meer heeft. Ook dit hebben we kunnen visualiseren met microscopie. Hiermee laten we zien dat *S. aureus* deze twee mechanismen voor het afschermen van het bacteriële oppervlak waarschijnlijk tegelijk kan gebruiken. Zo kan het de remming van fagocytose versterken in verschillende plasmaconcentraties en dus

verschillende plekken in het menselijk lichaam of in verschillende stadia van een infectie. Omdat *S. aureus* deze zeer effectieve mechanismen om fagocytose te ontwijken heeft ontwikkeld proberen wij mechanismen te ontwikkelen om fagocytose van de bacterie toch te kunnen verbeteren. Onlangs werd beschreven (Diebolder *et al.*, Science 2014) dat antilichamen, met name IgG, gebonden aan het oppervlak van tumorcellen interactie aangaan door middel van de Fc-domeinen. Hierbij laat één van de Fab-domeinen los van het oppervlak en worden er structuren van meerdere IgG's gevormd, waarbij zes antilichamen (een hexameer) de optimale vorm is (**figuur 3**). De zes antilichaam-bindende 'koppen' van het C1-complex passen perfect op deze hexameren. Hierdoor gaat het een zeer sterke binding aan en is er een hoge activatie van de opvolgende complementcascade. Met antilichamen die gemodificeerd zijn voor een sterkere of zwakkere Fc-interactie (en die dus meer of minder hexameren vormen) laten ze zien dat dit leidt tot een verhoogde of verminderde vorming van het MAC en opvolgende celdood. In **hoofdstuk 4** tonen we aan dat ook voor de antilichamen gebonden aan bacteriën (wij gebruikten vooral *S. aureus*) deze vorming van structuren van meerdere antilichamen erg belangrijk is. Door de hexameren op te heffen, met een klein eiwit dat de Fc-interacties verbreekt, laten we zien dat deze formatie essentieel is voor complementactivatie op het bacteriële oppervlak en voor de opvolgende opname door neutrofielen. We hebben antilichamen gebruikt die zich richten tegen onderdelen van de celwand van *S. aureus* en tegen het CP5 kapsel. Ook deze antilichamen zijn gemodificeerd om meer of minder hexameren te vormen. Hiermee tonen we aan dat complementactiviteit en fagocytose kunnen worden verlaagd maar ook verhoogd, wat zeer gunstig is voor implicaties in antilichaamtherapieën tegen *S. aureus*-infecties, en zeer waarschijnlijk ook bij andere bacteriële infecties.



Figuur 3. Schematische voorstelling van binding van C1 (blauw en taupe) aan oppervlak-gebonden antilichamen (groen) die Fc-interacties aangaan, waarbij één Fab-domein ongebonden is. In het bovenaanzicht is de optimale hexameer-structuur van IgG te zien.

Omdat neutrofielen, naast complementfactoren, ook direct de oppervlak-gebonden antilichamen kunnen herkennen door middel van Fc-receptoren, onderzochten we of de vorming van IgG-hexameren daar ook invloed op heeft. In **hoofdstuk 5** laten we zien dat ook Fc-receptor-gemedieerde fagocytose, dus in de afwezigheid van complementfactoren, beïnvloed wordt door hexameer-formatie. Wanneer antilichamen gebruikt werden die sterkere hexameren vormen werd ook de fagocytose door middel van Fc-receptor-interactie verhoogd. Daarmee tonen we aan dat fagocytose, in het lichaam gemedieerd door zowel complementreceptoren als Fc-receptoren op de neutrofielen, positief beïnvloed kan worden door het versterken van IgG-hexameren.

Ter conclusie: dit proefschrift laat zien dat *S. aureus* sterke mechanismen heeft ontwikkeld om fagocytose te omzeilen maar dat we met nieuwe antilichaam-mechanismen de opname door fagocyten toch kunnen versterken en zo mogelijk *S. aureus*-infecties beter kunnen bestrijden of voorkomen.

Dankwoord

Huh, is het gelukt? Mijn proefschrift is echt af! Ik kan het bijna niet geloven.
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En dan als laatst…….Lipase. Ha, staat hij toch in mijn boekje!

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P.S. please wear panties to my defense ☺.

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En als laatst, bedankt **laptop** dat je niet gecrasht bent tijdens dit stressvolle proces. Dat waardeer ik zeer! ☺

Curriculum vitae

Annemarie Kuipers was born on August 4, 1980, in Voorburg, the Netherlands. In 1999 she completed her secondary school education (Gymnasium) at the Interconfessioneel Makeblijde College in Rijswijk. After an additional year of chemistry at the Elckerlyc College in Den Haag, she started her studies in Biology and Medical laboratory sciences at the Hogeschool Leiden. In 2005 she performed an internship under the supervision of Dr. Kok van Kessel, on the interplay between evasion proteins of *Staphylococcus aureus* and neutrophil proteases, at the department of Medical Microbiology, University Medical Center Utrecht in the lab of Prof. dr. Jos van Strijp. After she obtained her Bachelor of Applied Sciences degree in 2006 she started as a research technician at the same department, working with Dr. Kok van Kessel. In 2012 she worked at the Helmholtz-Zentrum für Infektionsforschung in Braunschweig, Germany under the supervision of Dr. Manfred Rohde, for three months on Scanning Electron Microscopy of bacteria. After returning to the Netherlands in 2012, she started as a PhD candidate in the group of Dr. Suzan Rooijakkers, at the department of Medical Microbiology, University Medical Center Utrecht. During her PhD training she studied phagocytosis of staphylococci by neutrophils and the effects of staphylococcal evasion proteins and human antibodies on this process. The results of her research are described in this thesis and published in peer-reviewed scientific journals.

List of publications

In this thesis:

Kuipers A*, Ko YP*, Freitag CM*, Jongerius I*, Medina E, van Rooijen WJ, Spaan AN, van Kessel KP, Höök M, Rooijakkers SH**. Phagocytosis escape by a Staphylococcus aureus protein that connects complement and coagulation proteins at the bacterial surface.** *PLoS Pathog. 2013 Dec;9(12).*

Kuipers A*, Berends ET*, Ravesloot MM, Urbanus RT, Rooijakkers SH. **Bacteria under stress by complement and coagulation.** *FEMS Microbiol Rev. 2014 Nov;38(6):1146-71.*

Kuipers A, Stapels DA, Weerwind LT, Ko YP, Ruyken M, Lee JC, van Kessel KP, Rooijakkers SH. **The *S. aureus* polysaccharide capsule and Efb-dependent fibrinogen shield act in concert to protect against phagocytosis.** *Microbiology 2016; doi: 10.1099/mic.0.000293.*

Other publications:

Zhang X, Paganelli FL, Bierschenk D, Kuipers A, Bonten MJ, Willems RJ, van Schaik W. **Genome-wide identification of ampicillin resistance determinants in Enterococcus faecium.** *PLoS Genet. 2012 Jun;8(6):e1002804.*

Stemerding AM, Köhl J, Pandey MK, Kuipers A, Leusen JH, Boross P, Nederend M, Vidarsson G, Weersink AY, van de Winkel JG, van Kessel KP, van Strijp JA. **Staphylococcus aureus formyl peptide receptor-like 1 inhibitor (FLIPr) and its homologue FLIPr-like are potent FcγR antagonists that inhibit IgG-mediated effector functions.** *J Immunol. 2013 Jul 1;191(1):353-62.*

Berends ET, Dekkers JF, Nijland R, Kuipers A, Soppe JA, van Strijp JA, Rooijakkers SH. **Distinct localization of the complement C5b-9 complex on Gram-positive bacteria.** *Cell Microbiol. 2013 Dec;15(12):1955-68.*

Stapels DA*, Kuipers A*, von Köckritz-Blickwede M, Ruyken M, Tromp AT, Horsburgh MJ, de Haas CJ, van Strijp JA, van Kessel KP, Rooijakkers SH.**Staphylococcus aureus protects its immune-evasion proteins against degradation by neutrophil serine proteases.** *Cell Microbiol. 2015 Sep 29.*

* equal contribution