Complement evasion by *Staphylococcus aureus*

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Complement evasion by *Staphylococcus aureus*

Ontwijking van het complement system
door *Staphylococcus aureus*

(met een samenvatting in het Nederlands)

Proefschrift

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The most exciting phrase to hear in science, the one that heralds new discoveries, is not Eureka! (I found it!) but rather, “hmm... that’s funny....”

Isaac Asimov

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Chapter 1

General Introduction

Bacterial complement escape

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Innate immunity

The immune system protects the human body from invading organisms like viruses, parasites, and bacteria. It is composed of the innate and the adaptive immune system. In order to function properly, the immune system must recognize and quickly kill a diverse array of micro-organisms. Furthermore, the immune system must spare host tissues; therefore, it should be able to distinguish between self and non-self. The immune system uses cellular and non-cellular components to perform these tasks. The adaptive immune system is composed of T- and B-cells. The adaptive immune response is characterized by specificity and long-lasting memory, but its activation is rather slow. It can take several days to mount a fully active adaptive immune response. Therefore, the human body also needs the innate immune system which acts very rapidly[1]. The innate immune system consists of different elements including anatomic barriers like the human skin and mucous membranes. Also secretions, as tears and saliva, are important for our defense against microbes as they contain lysozyme, defensins, and cathelicidins that have antimicrobial activity[2-4]. When microbes cross the anatomic barriers, they will be identified by the innate immune system through recognition of evolutionary-conserved structures. Next to the activation of the complement system, which will be discussed in detail below, the cells of the innate immune system are also critical as these aggressive cells can kill and remove the uninvited guests within minutes. Cells important for this clearance are the polymorphonuclear leukocytes (neutrophils, eosinophils, and basophils) and mononuclear phagocytes (macrophages, monocytes, and dendritic cells)[1].

The immune cells can recognize invading microbes via pattern recognition receptors that recognize specific patterns on the microbial surface, the so-called pathogen-associated microbial patterns. These pathogen-associated microbial patterns are conserved among microbes and are not present on higher eukaryotic cells[5]. Therefore, only a limited number of receptors is needed to recognize a large range of microbes[6]. Examples of pathogen-associated microbial patterns are lipopolysaccharides, which are present on the surfaces of Gram-negative bacteria and are recognized by toll-like receptor 4, or formylated peptides, which are secreted by all bacteria and are recognized by the Formylated-Peptide Receptor (FPR).

Activation of the innate immune system results in an interaction of all components of this system. Additionally, there is a constant interaction between the innate and the adaptive immune system. For instance, antibodies or immunoglobulins (Ig) are produced by cells of the adaptive immune system but play an important role in the innate immunity response. Antibodies can neutralize microbial toxins, they activate the complement system, and they can direct the clearance of microbes via Fc receptors on phagocytic cells[7,8].

Complement

The complement system is a major mediator of the innate immune system, our first line of defense against invading micro-organisms[9,10]. It consists of more than thirty proteins found in plasma and on cell surfaces, and its activation results in a quick and effective defense against invading microbes. An important eradication strategy is the opsonization of foreign substances with C3b and iC3b, which marks them for uptake by neutrophils via complement receptors[11,12]. Furthermore, Gram-negative bacteria can be directly killed via the formation of C5b-9, the membrane attack complex (MAC), on the bacterial cell surface[13]. The complement system also forms a bridge between the innate and adaptive immune system because C3d, the degradation product of C3b and iC3b, facilitates antigen presentation to B-cells[14].

Activation routes of the complement system

The complement system comprises three different activation routes to recognize extrinsic substances (Figure 1): the classical (CP), the lectin (LP), and the alternative (AP) pathway. The CP is activated when the first component of the complement system, C1, binds to IgG or IgM molecules attached to a microbial surface. C1 is composed of C1q, C1r, and C1s, and the complex forms in the presence of Ca^{2+} in a 1:2:2 ratio. C1q is responsible for the binding of C1 to antibodies on the target surface. Binding of C1q to an activator leads to the autoactivation of the bound C1r, which can thereafter activate C1s. Activated C1s in turn then cleaves another complement protein C4, resulting in the formation of the anaphylatoxic peptide C4a and C4b[7,12,15,16]. The C4b molecule covalently binds to the bacterial surface due to the exposure of its internal thioester that reacts with hydroxyl (creating an ester bond) or amino group (creating an amide bond)[16]. Subsequently, C2 binds to surface-bound C4b, whereupon it is cleaved by activated C1s to form C3b2a a complex called the C3 convertase[12].

The LP is highly similar to the CP, and its activation also results in the formation of the C3 convertase C4b2a. However, the recognition molecules of the LP are mannan-binding lectin (MBL) and ficolins (L-, H-, or M-ficolins). These lectins are structurally similar to C1q, but they recognize microbial sugar patterns instead of immune complexes[17]. MBL and ficolins recognize neutral sugars (preferentially mannose, N-acethylglucosamine, and fucose) when presented in a repetitive manner as is the case on the surface of a range of microbes[18,19]. MBL and ficolins are associated with MBL-associated serine protease (MASP)-1, MASP-2, MASP-3, and a non-protease small MBL-associated protein (sMAP or MAP19)[17]. MASP-2 is the only protease known

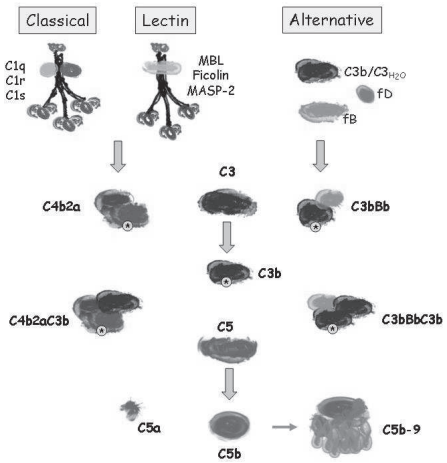

Figure 1: Schematic overview of the complement system

Complement activation can occur via three different pathways. The antibody-dependent CP starts when C1q in the C1q-C1r$_2$-C1s$_2$ complex recognizes antibodies that are bound to the microbial surface. In the LP, MBL and Ficolins recognize microbial sugar patterns and activate MASP-2. Both C1s and MASP-2 can cleave complement proteins C4 and C2 to generate the CP/LP C3 convertase: C4b2a. Within this complex, C4b is covalently (*) attached to the microbial surface. The AP C3 convertase (C3bBb) is generated after binding of fB to surface-bound C3b or fluid-phase C3(H_2O). FB is subsequently cleaved by fD to generate C3bBb. Both C3 convertases C4b2a and C3bBb cleave C3 into covalently bound C3b (*) and an anaphylatoxin C3a. C3b contributes to phagocytosis, antigen presentation and formation of C5 convertases, C4b2a3b and C3bBb3b. C5 convertases cleave C5 into an anaphylatoxin C5a and C5b, which forms a complex with complement proteins C6, C7, C8, and C9 to generate the MAC and mediate microbial lysis.

to be responsible for cleavage of C4 and C2 to generate the C2 convertase C4b2a[18]. Other less well-defined functions of MASPs are activation of the AP by direct cleavage of C3 by MASP-1[20] and activation of the coagulation system by MASP-2[21].

The AP is activated upon covalent attachment of C3b to bacterial surfaces via the CP and LP[12]. Factor B (fB) binds to both surface-bound C3b in the presence of Mg^{2+}, and the pro-convertase C3bB will be formed. FB bound to C3b becomes susceptible to proteolysis by factor D (fD) and is cleaved into the small fragment Ba and the big fragment Bb. The Bb fragment stays attached to C3b and the AP C3 convertase (C3bBb) is formed. Regulation of the convertase is achieved by fast C2a Bb dissociation from C4b and C3b respectively (60-90 seconds at 37°C)[22]. Next, C2a and Bb cannot rebind the C4b or C3b molecule after dissociation[23,24].

C3 convertase

As the initiation of all three convertase pathways results in the formation of C3 convertases (C4b2a for the CP/LP and C3bBb for the AP), there enzyme complexes fulfill a central role in the complement cascade. C3 convertases are bimolecular complexes that only have a short lifespan of 1-2 minutes because C2a and Bb dissociate fast from the complex in an irreversible manner[24]. The function of C3 convertases is the cleavage of C3 into C3a and C3b. C3 is the most abundant complement protein in serum (its concentration is 1 mg/ml), and its cleavage to C3b and C3a by C3 convertases is an important step in the complement cascade[25,26]. C3a is a small, fluid-phase chemoattractant, while C3b covalently attaches to the microbial surface via its thioester. Both C3b and its degradation product iC3b mark microbes for efficient uptake by phagocytes upon recognition by complement receptor (CR) 1 and CR3, respectively[9,10]. Further degradation of iC3b by factor H (fH) and I (fI) results in the formation of C3d, which is recognized by CR2 on B-cells, supporting antigen presentation[26]. The degradation of C3 and the function of CR1-3 will be discussed in more detail below.

C5 convertase

In addition to its role in phagocytosis, C3b molecules are also needed for initiation of the terminal pathway of the complement system. The binding of an additional C3b molecule to the already formed C3 convertases results in the formation of C5 convertases: C4b2aC3b (CP/LP) and C3b2Bb (AP)[27,28]. These convertases preferentially cleave C5 instead of C3 and form two C5 split products (C5a and C5b). The simplest form of a C5 convertase is the C3 convertase of the AP. Although the catalytic rate of the C5 cleavage by this enzymatic complex is similar to natural surface-bound C5 convertases, its affinity for C5 is very weak, and, based on its C3 and C5 cleavage properties, it is estimated that this C3 convertase will cleave 9000 C3 molecules for every C5 molecule[29]. The natural but more complex forms of C5 convertases are enzymes with an extra C3b molecule attached on or near C3 convertases[30-33]. It is still unclear whether C5 convertases are composed of C4b-C3b or C3b-C3b dimers that are covalently attached to each other, or whether C5 convertases are formed after an increased concentration of C3b is deposited on the bacterial surface, which results in the presence of an extra C3b molecule close to the formed C3 convertase[33-36].

As already described above, C5 convertases cleave C5 into C5a and C5b. The C5 cleavage product C5a is a very potent chemotactic factor which recruits and activates phagocytes by binding to the C5a receptor (C5aR). C5b, on the other hand, forms a soluble complex with C6, and subsequent binding of C7 results in hydrophilic to amphiphilic transition of the C5b-C6-C7

complex and induces expression of a metastable site through which C5b-7 is inserted into target lipid bilayer membranes. Subsequent incorporation of C8 and multiple C9 molecules allows the complex to traverse lipid bilayers, resulting in the formation of complete transmembrane channels that induce osmotic lysis of the targeted cell. This so-called MAC can directly kill Gram-negative bacteria, while Gram-positive bacteria are protected by their thick cell wall[37].

Complement regulators

As host tissue can also be targeted for damage by the complement system, the complement cascade is tightly regulated by several fluid-phase and cell-bound molecules. The activity of the convertases is regulated by a large group of proteins called regulators of complement activation. Fluid-phase molecules include fH and factor H-like protein 1 that accelerate decay of the AP convertase, while C4-binding protein dissociates the CP/LP convertase. Host cells express membrane-bound CR1 and decay-accelerating factor (DAF) that dissociate C4b2a. Furthermore, membrane co-factor protein and most of the above-described convertase regulators are also cofactors for fI-mediated degradation of C4b and C3b[14]. The CP/LP enzymes C1r, C1s, and MASPs are controlled by the serine protease inhibitor termed C1 Inhibitor[38]. The formation of the MAC is regulated by molecules that prevent insertion of C5b-7 into lipid membranes; these molecules include membrane-bound CD59 and fluid-phase vitronectin and clusterin[39]. Finally, the AP C3 convertase is positively regulated by properdin, a multimeric protein that increases the stability of the complex[40].

Structure and degradation of C3

C3 can be seen as the key protein in the complement system as all complement pathways converge in the cleavage of C3, resulting in coverage of target surfaces with C3b that is important for phagocytosis, C5a generation, and activation of the adaptive immune response[10,26-28]. C3 is a 187 kDa protein that belongs to the α2-macroglobulin family and evolutionary emerged over 700 million years ago[41]. The C3 protein found in serum consist of a α chain and an β chain, together forming 13 domains (Figure 2). The C3 protein cannot bind to bacterial surfaces, and its structural stability is contributed to the MG8 domain[42]. Cleavage of C3 by C3 convertases occurs at position Ser726-Arg727. Upon cleavage, the ANA domain (C3a, 9 kDA) is released, and C3b (177 kDa) is formed[25,41,42] C3b is composed of 12 domains and can bind covalently to the bacterial surface via a highly active thioester[41]. This thioester is protected in the native C3 molecule and only gets exposed after C3 is cleaved[25]. Furthermore, cleavage of C3 into C3b results in large rearrangements of the α chain, which leads to the exposure of several binding sites for fB, fH, fI, properdin, DAF, and CR1[41,42].

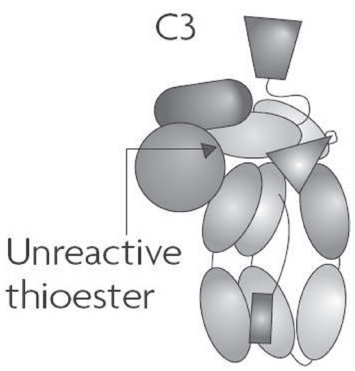

Figure 2: Schematic representation of the C3 molecule
C3 is composed of 13 separate domains. The thioester, which is important for surface binding, is unreactive in the C3 molecule as it is hidden within the molecules. Figure adapted from Gros et al., 2008[93].

The innate immune system designed several mechanisms to prevent the complement system to be over reactive, especially to prevent damage to human cells. The regulators of complement activation are already described above, but here their influence on degradation of C3b will be discussed. Degradation of C3b begins with the cleavage of the α chain by fI and cofactors such as fH or CR1, resulting in the formation of iC3b (177 kDa), which consist of three chains. The α chain can be cleaved for a second time resulting in the release of C3f (2 kDa) and the remaining iC3b (175 kDa). The third cleavage in the remaining iC3b results in the release of C3c (135 kDa) from the target bound C3dg (40 kDa)[25] (Figure 3)[43-45].

Complement Receptors

C3b and its degradation products iC3b and C3dg can be recognized by CR1, 2, 3, 4, and Complement Receptor of the Immunoglobulin superfamily (CRIg). CR1 and 2 belong to the regulators of complement activation family, while CR3 and CR4 also functions as adhesive molecules termed integrins[46-48]. CRIg is a type 1 transmembrane Ig superfamily member[49] (Figure 4).

CR1 (CD35) is a large type 1 transmembrane glycoprotein which is composed mainly of tandemly-arranged modules of 60-70 amino acids long known as complement control protein repeats (CCPs)[50]. CR1 is expressed on erythrocytes, macrophages, neutrophils, B cells, follicular dendritic cells, and a subset of T cells[51]. CR1 can bind C3b and C4b well, and with a lower affinity it can bind iC3b and C3dg. Furthermore, as already explained above CR1 is in important cofactor for fI. Three important active sites are present in the CR1 molecule. Site one is located in CCPs 1-3, binds C4b and accelerates the decay of the C3 convertases. Site two and three are located in CCPs 8-10 and 15-17, respectively, and both sites bind to either C3b or C4b (Figure 4a). These sites have a high co-factor activity for fI[14,50]. CR1 is a very important receptor on neutrophils for phagocytosis of C3b-coated microbes[50].

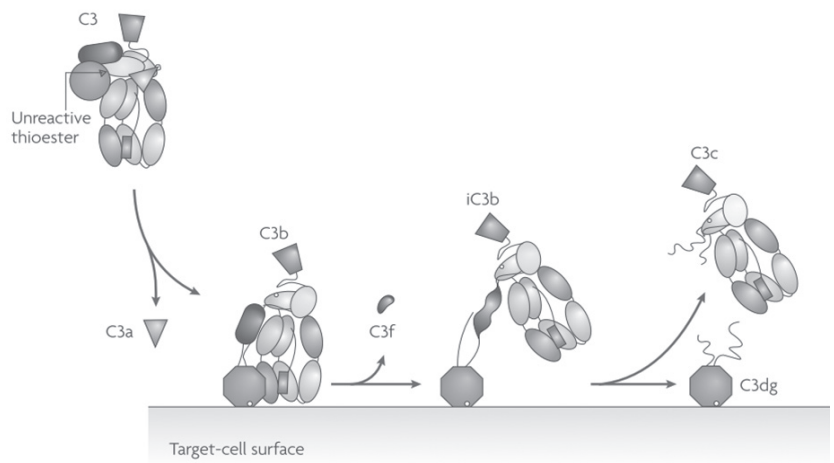

Figure 3: Schematic representation of C3b degradation
C3 is cleaved by C3 convertases resulting in the formation of C3b, which covalently binds to the target surface via the thioester, and C3a, which is released as a chemoattractant. Degradation of C3b by fI and cofactors results in sequential formation of iC3b,C3f, C3c, and C3dg. Figure adapted from Gros et al., 2008[93].

Similar to CR1, CR2 belongs to the RCA family and it is a type 1 membrane glycoprotein. CR2 is found on mature B cells, follicular dendritic cells, epithelial cells, and some T cells. Depending on alternative splicing, CR2 is composed of 15 or 16 CCPs. It has a transmembrane region and a short cytoplasmatic tail. CR2 recognizes the C3 degradation products iC3b, C3dg, and C3d[52]. Mutation studies show that the active site for binding C3 degradation product is positioned in the first two CCPs[53,54] (Figure 4). CR2 links the innate and adaptive immune system since binding of an antibody coupled to C3d enhances B-cell activation up to a thousand fold[11,55-57]. CR2 binds the C3dg molecule, while the antigen binds to the B-cell receptor (BCR). Because of its short cytoplasmatic tail, CR2 needs his co-receptors CD19 and CD81 for signaling. The cytoplasmatic tail of CD19 consists of nine tyrosine residues in the cytoplasmatic domain which become phosphorylated after activation[58]. Coupling of CR2 and BCR leads to the coupling between BCR and CD19, which enhances B-cell activation[55,59] (Figure 5).

CR3 (CD11b/CD18) is a β2 integrin, and is expressed exclusively on leukocytes. CR3 is an adhesion and recognition receptor for both endogenous ligands (including iC3b) as well as an array of microbial molecules. It is known as the phagocytic receptor on phagocytes[46]. CR3 can bind to iC3b but also cooperates with CR1. Binding of CR3 to iC3b activates the receptor which results in activation of other receptors such as CD14, FcγRII, and CR1 together leading to phagocytosis of the marked microbe[46].

CR4 (CD11c/CD18) also known as p150,95 is mainly expressed on tissue derived macrophages where it is found to be the major C3 component binding receptor. CR4 has the same function as CR3; it mainly binds to iC3b and is important for clearance of opsonized particles[11,48,60].

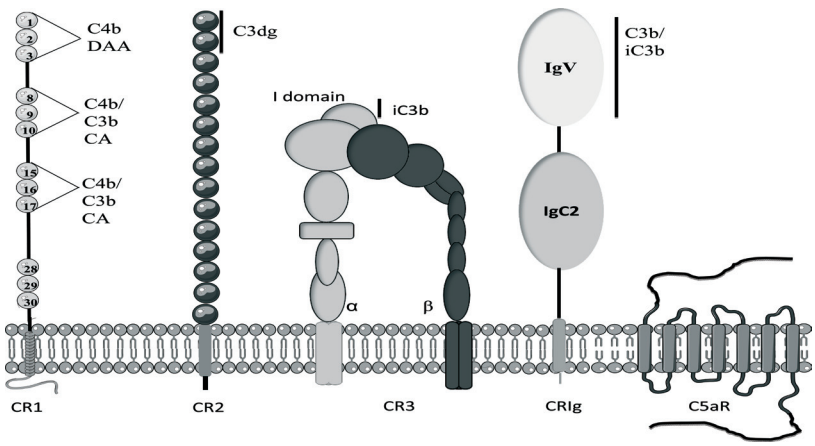

Figure 4: Schematic representation of complement receptors
CR1 is large type 1 transmembrane Ig superfamily member composed of 30 CCPs. CCP1-3 are important for C4b binding and Decay Accelerating Activity (DAA), while CCP 8-10 and CCP 15-17 bind C3b and C4b in addition both CCP domain have cofactor activity (CA) for fI[50]. CR2 is composed of 15 or 16 CCPs and the first two CCPs are involved in C3dg binding[55,56]. CR3 is a β2 integrin, the I domain positioned at the α-chain is responsible for the iC3b binding[46]. CRIg is composed of an IgV binding domain that binds C3b and iC3b, and an IgC2 domain[49]. The C5aR belongs to the group of G-protein coupled receptors a large family of receptors with seven helical membrane-spanning regions connected by six extramembrane loops[63]. The extracellular N-terminus interacts with the receptor specific ligands, in this case C5a, the intercellular C-terminus is involved in G-protein recognition and activation.

Recently, a new complement receptor has been identified that was termed CRIg. CRIg is uniquely expressed on tissue resident and sinusoidal macrophages. CRIg is the only complement receptor known on liver Kupffer cells. It is important for clearance of opsonized targets and can be found in two forms. The longer form of human CRIg (huCRIg(L)) encodes both the V- and the C2 type terminal Ig domains, while the short form (huCRIg(S)) encodes a single V type Ig domain[49]. The V type Ig domain is responsible for binding to IgM. CRIg recognizes C3b, iC3b, and methylated C3. Soluble CRIg can inhibit the C3 and the C5 convertase of the AP since it prevents binding of C3 and C5 to the AP convertases[61].

Another complement receptor that binds C5a instead of a degradation product of C3 is the C5aR. The C5aR is found on myeloid cells, including neutrophils, basophils, eosinophils, monocytes and macrophages[62]. The C5aR belongs to the group of G-protein coupled receptors a large family of receptors with seven helical membrane-spanning regions connected by six extramembrane loops[63]. The extracellular N-terminus interacts with the receptor specific ligands, in this case C5a, the intercellular C-terminus is involved in G-protein recognition and activation.

Staphylococcus aureus and its immune evasion molecules

The complement system is efficient in recognition and destruction of a variety of micro-organisms. However, sometimes microbes can escape recognition through production of several modulators. *Staphylococcus aureus* is an example of pathogen that can evade the complement system and cause severe disease. *S. aureus* is a Gram-positive bacterium which can be distinguished from other staphylococcal species on basis of the gold pigmentation of its colonies and positive results of coagulase, mannitolfermentation, and deoxyribonuclease tests. Staphylococcal disease was already reported and published in 1882[64], and more than 100 years later *S. aureus* remains a dangerous pathogen for humans[65]. *S. aureus* causes a number of community- and hospital-derived infections, ranging from uncomplicated wound infections and light food-poisoning to severe diseases as bacteremia or endocarditis[65]. *S. aureus* expresses a lot of virulence factors that contribute to its survival in the human host. Secretion of numerous toxins and superantigens can result in for instance toxic shock syndrome and food-poisoning. Superantigens affect the immune system by binding to histocompatibility complex class II and T cell receptors. This binding results in a specific activation of T cells that leads to the release of several cytokines, interleukins, and interferon-γ. Moreover, *S. aureus* produces proteins such as lipase and protease that can directly destroy tissue, which may facilitate the spread of its infection[65]. Only recently it was shown that *S. aureus* also expresses a large number of proteins that specifically impair the effectiveness of the innate immune system[66-68]. These include factors that modulate antimicrobial peptides and phagocytic cells but also complement modulators.

The first anti-opsonic molecule identified in *S. aureus* was Staphylococcal protein A (SpA) (42 kDA), a surface protein that can also be released in the extracellular milieu[69]. By binding the Fc part of IgG molecules, SpA covers the bacterial surface with IgG molecules in a manner that prevents recognition by Fc-receptors on phagocytes. This way, SpA blocks Fc-receptor mediated phagocytosis. Furthermore, since SpA interferes with C1q binding, it prevents CP complement fixation as well[70,71].

The staphylococcal cell wall-associated protein Staphylococcus aureus binder of IgG (Sbi) (45 kDa) is also involved in immunoglobuling binding[72]. The Sbi extracellular region (Sbi-E) consists of four major domains including two IgG-binding regions. Recent data have indicated that the other two domains have complement-inhibitory functions since they bind

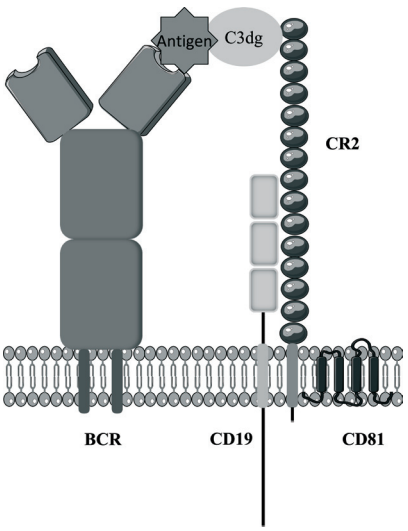

Figure 5: Schematic representation of CR2 and BCR coupling
(b) CR2 has a short cytoplasmatic tail and needs CD19 and CD81 for signalling. CR2 binds the C3dg molecule, while the antigen binds to the B-cell receptor (BCR). The cytoplasmatic tail of CD19 consists of nine tyrosine residues in the cytoplasmatic domain which become phosphorylated after activation[58]. Coupling of CR2 and BCR leads to the coupling between BCR and CD19, which enhances B-cell activation[55,59].

C3 via the thioester-containing C3dg fragment and the C3a anaphylatoxin domain thereby activating C3 fluid phase leading to consumption of the complement system. It is also describe that Sbi forms a tripartite complex with C3b and factor H, thereby blocking the activation of the AP[73-75].

Another way for *S. aureus* to inhibit phagocytosis is through the production of staphylokinase (SAK) (16 kDa)[76]. SAK targets plasminogen to the bacterial surface and activates it into plasmin. Plasmin can cleave two major opsonic molecules, IgG (at the hinge region) and C3b[45,77]. It was shown that the conversion of plasminogen to plasmin by SAK leads to removal of IgG and C3b on the bacterial surface. Thus, SAK impairs both IgG and complement dependent phagocytosis by human neutrophils[76].

Staphylococcal superantigen-like (SSL) protein 7 is a 23 kDa protein that belongs to a family of 11 different SSL's that are clustered on staphylococcal pathogenicity island 2 (SapIn2)[78,79] (pathogenisity islands and mobile elements will be discussed in detail below). SSL7 binds to IgA but also prevents formation of the MAC via specific binding to C5[79]. SSL7 also prevents the formation of C5a, thereby inhibiting neutrophils migration *in vivo* (unpublished data, Bestebroer). The latter effect seems more important for clearance of *S. aureus* as its thick cell wall renders it resistant to MAC-mediated lysis.

The importance of inhibiting C5a-mediated responses was already indicated by another staphylococcal protein called the chemotaxis inhibitory protein of *S. aureus* (CHIPS) (14kDa)[80]. This extracellular protein specifically binds two major chemotactic receptors on neutrophils, the C5aR and the FPR[81]. By binding these receptors, CHIPS effectively blocks neutrophil chemotaxis towards C5a and formylated peptides (e.g. fMet-Leu-Phe)[80]. It was shown that the first and the third residues of the N-terminus of CHIPS is important for the inhibition of the FPR, while mutation of these residues had no affect on the properties to inhibit the C5a receptor. This indicates that in this small CHIPS molecule two active sites are present[82].

Finally, *S. aureus* secretes a number of proteins that specifically block convertases activity. The first-described convertase inhibitor is Staphylococcal Complement Inhibitor (SCIN) (10 kDa), an secreted protein that binds C3 convertases and interferes with all complement

pathways[83]. SCIN binding to surface-bound C3 convertases has two major consequences. Firstly, the binding of SCIN to C4b2a and C3bBb impairs the enzymatic activity of the convertases. Secondly, SCIN stabilizes C3 convertases which impairs the formation of new convertases. These actions result in very potent inhibition of phagocytosis and C5a production[83,84].

Recently, the stabilization property of SCIN on the AP convertase was used to solve the structure of the AP convertase (C3bBb) in complex with SCIN. This structure revealed that stabilization of C3bBb is essential for inactivation of the enzyme since SCIN inhibits the swinging of Bb by fixing the Bb molecule on C3b[85]. However, the downside of stabilizing C3bBb on bacterial surfaces seemed to be that C3b molecules within complexes could still trigger phagocytosis. In chapter 2 we will uncover that SCIN circumvents phagocyte responses by forming dimeric convertase complexes that cannot be recognized by complement receptors. Next to the production of SCIN, we found that *S. aureus* also produces two homologues of SCIN. The function of these homologues will be discussed in chapter 3.

Another molecule that possibly influences complement activation is Extracellular fibrinogen binding protein (Efb), which was first found to bind to fibrinogen[86-88]. Next to the binding to fibrinogen, it has been shown that Efb modulates platelet function and inhibits platelet activation[89]. Furthermore, it has been shown that an Efb knock-out strain was not able to induce severe wound infection in contrast to the wild type strain[90]. Recently, it has been shown that Efb has yet another function: it can bind to complement component C3[91]. Efb binds to the α-chain of C3, resulting in the inhibition of the CP and the AP of complement. Efb binds specifically to the C3d domain of C3 with its C-terminal part, while the N-terminal part of Efb is responsible for the binding to fibrinogen[91]. The structure of the C-terminal domain of Efb (Efb-C) together with C3d showed that amino acid R131 and N138 in Efb-C are important for its binding to C3d[92]. Furthermore, it has been shown that Efb-C preferably binds to native C3 and thereby inhibits the formation and deposition of C3b on the microbial surface[92]. A homolog of Efb-C was found and named Efb homologous protein (Ehp). We have identified the same homolog of Efb, but have named it Extracellular complement binding protein (Ecb) (chapter 3).

Aim of the study

In this thesis, the mechanism of action for complement evasion molecules by *S. aureus* will be investigated. For this purpose, the main focus will be put on Efb and Ecb and their role in complement evasion of *S. aureus*. Not only will the human specificity of these molecules be investigated, but also their mechanism of action, both *in vitro* as *in vivo*, will be examined. Finally, the relevance of secretion of complement inhibitory molecules for *S. aureus* pathogenesis *in vivo* will be explored.

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Chapter 2

Staphylococcal complement inhibitor modulates phagocyte responses by dimerization of convertases

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ABSTRACT

The human pathogen *Staphylococcus aureus* produces several complement evasion molecules that enable the bacterium to withstand the host immune response. The human-specific Staphylococcal Complement Inhibitor (SCIN) blocks the central C3 convertase enzymes that trigger critical complement functions as C3b deposition, phagocytosis, and C5a generation. SCIN effectively blocks the conversion of C3 by Alternative Pathway C3 convertases (C3bBb) but also induces dimerization of these enzymes. Here, we show that formation of dimeric convertases by SCIN is important for *S. aureus* immune evasion since it modulates complement recognition by phagocytic receptors. Dimeric, but not monomeric, SCIN-convertases showed an impaired binding to Complement Receptor 1 and the Complement Receptor of the ImmunoGlobulin superfamily. The dimerization site of SCIN is essential for its strong anti-phagocytic properties. These studies provide critical insights into the unique immune evasion strategies employed by *S. aureus*.

INTRODUCTION

The human complement system is important to combat invading microorganisms. Its activation leads to coverage of target surfaces with C3b, resulting in phagocytosis and formation of C5 convertases which generate C5a that is important for chemotaxis of neutrophils towards the site of infection[1-3]. The complement system comprises three pathways; the Classical Pathway (CP), the Lectin Pathway (LP), and the Alternative Pathway (AP). Activation of these pathways results in formation of C3 convertases, C4b2a (CP/LP) and C3bBb (AP), which cleave C3[4-6]. Cleavage of C3 into C3b leads to remarkable conformational changes in the C3b molecule, allowing covalent binding of its thioester-containing domain to the target surface and exposure of essential binding sites for complement receptors on phagocytic cells and complement regulators[7,8]. C3b also supports the formation of C5 convertases, C4b2aC3b (CP/LP) and $C3b_2Bb$ (AP), which cleave C5 to generate the chemoattractant C5a and C5b that is important for the formation of the membrane attack complex[9,10].

Phagocytosis of microbes is an essential host defense to clear pathogens. Phagocytic cells, including neutrophils, macrophages, and Kupffer cells, express several complement receptors that recognize bacterium-bound C3b or iC3b. Formation of iC3b occurs after cleavage of deposited C3b by factor I (fI) together with co-factors factor H (fH) or Complement Receptor (CR) 1. Neutrophils express CR1 and CR3, while macrophages express CR3 and CR4. The Complement Receptor of the ImmunoGlobulin superfamily (CRIg) is exclusively found on liver Kupffer cells[11-13]. All complement receptors recognize C3b or iC3b, though through distinct binding sites[8,14-16].

Staphylococcus aureus is an important human pathogen that causes community- and hospital-derived infections ranging from uncomplicated wound infections and light food-poisoning to severe diseases as bacteremia or endocarditis. Emergence of methicillin-resistant strains in both hospitals and the community makes *S. aureus* an enormous threat to public health[17]. *S. aureus* has evolved several mechanisms to impair the human immune system, allowing the bacterium to survive and cause infections in the host. Several of its excreted molecules such as the human-specific Staphylococcal Complement INhibitor (SCIN) inhibit complement activation[18,19]. SCIN effectively modulates various functions of the complement system by inhibiting C3 convertases[20]. Our recent crystal structure of the AP convertase (C3bBb) in complex with SCIN demonstrated that stabilization of C3bBb is essential for inactivation of the enzyme[21]. However, the downside of stabilizing C3bBb on bacterial surfaces seems that C3b molecules within complexes could still trigger phagocytosis. In this study we uncover that SCIN circumvents phagocyte responses by forming dimeric convertase complexes that cannot be recognized by complement receptors.

MATERIALS AND METHODS

Protein expression and purification

C3 was purified from freshly isolated human plasma, and C3b was generated as described[21]. Preparation of recombinant SCIN was previously described[20]. In the SCINΔC3b$_2$ mutant, residues 5-8-61-64-67-68 were exchanged for alanines. FB was purified from human plasma as described previously[21], and fD, fH, fI and iC3b were obtained commercially (Calbiochem). Recombinant CR1 was a kind gift from Prof. John Atkinson (Washington University, St. Louis), recombinant CRIg-S, the short form of CRIg which binds C3b and iC3b, was obtained from Genentech, Inc.

Cell lines and maintenance

A Chinese hamster ovarian (CHO) cell line expressing CR1 (CR1-CHO) and a control R-CHO cell line were obtained from Prof. John Atkinson[22]. Cells were maintained in Ham's F12 medium (Gibco) with 10% FCS, 10 µg/ml gentamycin and 500 µg/ml neomycin (Gibco). A Jurkat cell line expressing CRIg (Jurkat-huCRIg) and Jurkat cells (negative control)[12] (Genentech, Inc) were maintained in RPMI 1640 medium containing 10% FCS, 10 µg/ml gentamycin and 500 µg/ml neomycin.

Convertase inhibition assay

C3 (250 nM), C3b (50 nM), fB (500 nM), fD (500 nM) and SCIN or SCINΔC3b$_2$ (2 µM) were incubated in HBS-Mg (Hepes-buffered Saline, 20 mM Hepes, 140 mM NaCl, 2.5 mM $MgCl_2$, pH 7.4) for 10 minutes at room temperature and C3 conversion was analyzed by SDS-PAGE under reducing conditions[21].

Convertase binding assays

Fluid-phase SCIN-convertases were prepared as described previously[21]. In short, 100 µg/ml C3b, 50 µg/ml fB, 5 µg/ml and 10 µg/ml his-SCIN or his-SCINΔC3b$_2$ were incubated in HBS-Mg for 1 hour at 4ºC after which the SCIN-convertases were pulled down by incubation with magnetic cobalt beads (Dynabeads Talon, Invitrogen) for 1 hour at 4ºC. Beads were washed three times with cold PBS, 0.05% Tween and complexes were eluted with PBS containing 50 mM EDTA. The amount of C3b present in SCIN-convertases was analyzed using SDS-PAGE. Results were excluded when the amount of C3b molecules in the SCIN- or SCINΔC3b$_2$-convertases were different from the amount of soluble C3b.

Microtiter plates (Greiner Bio one) were coated overnight with 5 µg/ml anti-C3c WM1 (American Type Culture collection[23]), recombinant CR1 or recombinant CRIg-S. Plates were blocked with PBS, 0.1% Tween and 4% BSA for 1 hour at 37ºC. Plates were washed three times between every step with PBS with 0.1% Tween. Soluble C3b (60 µg/ml), fluid phase SCIN- and SCINΔC3b$_2$-convertases were added for 1 hour at 4ºC where after binding was detected using PO-conjugated anti-C3 antibodies (1:5000) (Protos Immuno Research). Data were analyzed by a two-tailed unpaired student's t-test. Binding of soluble C3b or SCIN-convertases to cells expressing CR1 or CRIg was studied by incubation of various concentrations of fluid phase convertases or 60 µg/ml C3b to 50 µl cells (5x10^6 cells/ml) for 30 minutes at 4ºC. Cells were washed with RPMI containing 0.05% human serum albumin (HSA) and binding was detected using FITC conjugated anti-C3 antibodies (Protos Immuno Research) and flow cytometry. Cells were gated on basis of forward and side scatter properties. We analyzed the fluorescence intensity of 5000 cells and the mean FL was calculated using BD CellQuest Pro software.

Convertases stabilization assays

Convertase stability on bacterial surfaces was analyzed by incubation of *S. aureus* strain Wood 46 (5x10^6 cfu/ml) with 10% human serum in the presence or absence of 10 µg/ml SCIN or SCINΔC3b$_2$ for 30 minutes at 37ºC. Bacteria were washed, dissolved in PBS and left at 37ºC for several decay periods. After decay, bacteria were washed again and boiled in sample buffer. Bb formation was analyzed by western blotting using anti-fB antibodies (Quidel).

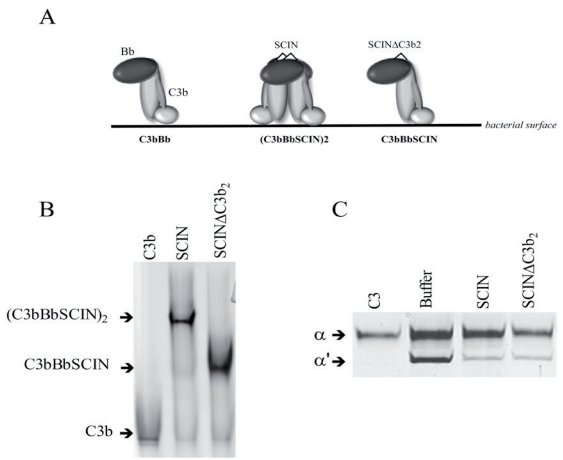

Figure 1: Convertase dimerization by SCIN is not necessary for convertase inhibition
(a) Cartoon representation of the active C3 convertase (C3bBb, left) and the inhibited convertase in the presence of SCIN (dimeric SCIN-convertase, middle) or the SCIN dimerization mutant (monomeric SCIN-convertas, right) (b) Native gel electrophoresis of AP C3 convertases formed in the presence of wild-type SCIN or SCINΔC3b$_2$. C3b, fB, fD were incubated with his-SCIN or his-SCINΔC3b$_2$ and purified by cobalt beads (c) C3 conversion by fluid-phase C3 convertases. C3 is incubated with C3b, fB and fD in the presence or absence of SCIN or SCINΔC3b$_2$, and C3 conversion was analyzed by SDS-PAGE under reducing conditions. Panels b and c are representative figures of three separate experiments.

C3b cleavage by fH and fI

Purified C3b (100 µg/ml), SCIN- and SCINΔC3b$_2$-convertases were incubated with 15 µg/ml fH and 5 µg/ml fI for 2 hours at room temperature. C3b conversion into iC3b was analyzed by SDS-PAGE under reducing conditions and silverstaining.

Complement deposition and CR1 binding to the bacterial surface

C3b deposition was performed by incubation of *S. aureus* strain Wood 46 with 0, 10 or 30% human serum in HBS-MgEGTA (Hepes-buffered Saline, 20mM Hepes, 140mM NaCl, 5 mM $MgCL_2$, 10 mM EGTA) for 30 minutes at 37°C in the presence or absence of SCIN or SCINΔC3b$_2$ (both at 10µg/ml). After washing, surface-bound C3b was detected with FITC-conjugated (Fab')$_2$ anti-human C3 antibody (Protos Immunoresearch, San Francisco) and flow cytometry. Bacteria were gated on basis of forward and side scatter properties. We analyzed the fluorescence intensity of 10.000 bacteria and the mean FL was calculated using BD CellQuest Pro software. To study surface-bound iC3b deposition, *S. aureus* strain Wood 46 ($5x10^6$ cfu/ml) was incubated with 30% human serum or fI-deficient serum in HBS-MgEGTA buffer for 30 minutes at 37°C in the presence or absence of SCIN or SCINΔC3b$_2$ (both at 3 or 10 µg/ml). FI-deficient serum was prepared by passing 1 ml of human serum through a $CoCl_2$ column loaded with anti-fI antibody OX-21[24,25] where after the fI deficiency was checked by western blotting. After incubation, bacteria were washed and boiled in sample buffer and iC3b formation was analyzed by immunoblotting using FITC-conjugated (Fab')$_2$ anti-human C3 followed by peroxidate-labeled donkey anti goat antibodies (Santa Cruz). To investigate CR1 binding to the bacterial surface, bacteria were opsonized in 30% human serum in the presence of SCIN or SCINΔC3b$_2$ (0, 1 or 3 µg/ml). After washing 10 µg/ml recombinant CR1 was added and detected with a PE labeled anti-CD35 and flow cytometry. Bacteria were gated on basis of forward and side scatter properties. Fluorescence intensity of 10.000 bacteria and the mean FL was calculated using BD CellQuest Pro software. Data were analyzed using a two-tailed unpaired student's T-test.

Phagocytosis

Phagocytosis was performed as described previously[20]. Shortly, FITC-labeled *S. aureus* strain KV27 was incubated with human serum for 2 minutes in the presence or absence of 10μg/ml SCIN or SCINΔC3b$_2$ at 37°C after which freshly isolated neutrophils were added for 15 minutes. Phagocytosis was analyzed by flow cytometry. Cells were gated on basis of forward and side scatter properties. Fluorescence intensity of 10.000 gated neutrophils was determined and the mean FL was calculated using BD CellQuest Pro software.

RESULTS

Dimerization of convertases by SCIN is not essential for inhibition of C3 conversion

The structure of C3bBb in complex with SCIN revealed that SCIN has a dimerization site that enables generation of dimeric (C3bBbSCIN)$_2$) instead of monomomeric (C3bBbSCIN) convertases[21]. To study the role of convertase dimerization by SCIN, we constructed a SCIN mutant (SCINΔC3b$_2$) that only forms monomeric convertases by replacing all residues involved in dimerization into alanines (Figure 1a). This is a more precise mutant than the previously described SCIN mutant ChC3b2 in which we exchanged residues 1-13, 59-61, 64-65 and 67-68 with corresponding residues from ORF-D, not only mutating the binding residues but other

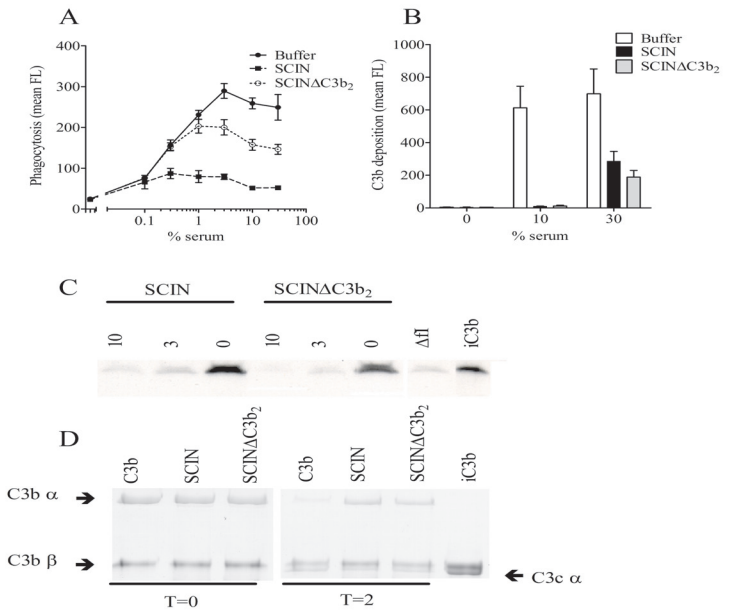

Figure 2: Convertase dimerization by SCIN is important for phagocytosis inhibition

(a) Phagocytosis of *S. aureus* strain KV27 by freshly isolated neutrophils in the presence of normal human serum 10 μg/ml SCIN or SCINΔC3b$_2$. (b) C3b deposition via the AP on *S. aureus* in the presence of SCIN or SCINΔC3b$_2$ (both at 10μg/ml). (c) iC3b formation on the bacterial surface in the presence of SCIN or SCINΔC3b$_2$ (both at 0, 3 or 10 μg/ml) or in fI-deficient serum (ΔfI). iC3b formation was analyzed by immunoblotting using an anti-C3 antibody. (d) iC3b formation in fluid-phase analyzed by SDS-PAGE under reducing conditions. C3b (100 μg/ml) was incubated with fH (15 μg/ml) and fI (5 μg/ml) at room temperature and iC3b formation was measured after 2 hours (T=2); T=0 shows the amount of C3b molecules at time point zero. Panels a and b represent the mean values ± SEM of three separate experiments, while c and d are representatives of three separate experiments. See supplementary figure 2 for representative histograms.

residues as well[21]. SCINΔC3b$_2$ forms stable monomeric convertase enzymes (figure 1b) that have the same half life as dimeric SCIN convertases (4 hours at 20°C, data not shown, 21). To investigate whether dimerization of AP convertases by SCIN is necessary for the inhibition of C3 conversion by convertases in fluid phase, we incubated C3 with C3b, fB, and fD in the presence or absence of SCIN or SCINΔC3b$_2$. Figure 1c shows that SCINΔC3b$_2$, which forms monomeric convertases, inhibits C3 conversion equally well as wild-type SCIN, which induces dimeric convertases.

SCIN-induced dimerization of convertases is important for inhibition of phagocytosis

Although wild-type SCIN and SCINΔC3b$_2$ inhibit C3 conversion equally in fluid phase conditions, this may be different on the bacterial surface. Therefore we analyzed phagocytosis of *S. aureus* by incubating the bacteria with serum and freshly isolated neutrophils in the presence of SCIN or SCINΔC3b$_2$. We observed that SCINΔC3b$_2$ had a markedly reduced ability to block phagocytosis as compared to SCIN. Wild-type SCIN completely inhibits phagocytosis, while SCINΔC3b$_2$ only inhibits by less than 50% (figure 2a).

As phagocytic uptake of bacteria by neutrophils is C3b and iC3b dependent, the deposition of these molecules on the bacterial surface was studied. Identical to our findings for fluid-phase C3 conversion, no difference in C3b deposition on the bacterial surface was found between SCIN and SCINΔC3b$_2$ (figure 2b). Next to that, we observed that iC3b was not formed on the bacterial surface in the presence of SCIN or SCINΔC3b$_2$ (figure 2c), suggesting that SCIN protects C3b from degradation by fH and fI. We prepared soluble SCIN-convertases by incubating C3b, fB, fD and his-SCIN or his-SCINΔC3b$_2$ and purified them using cobalt beads.

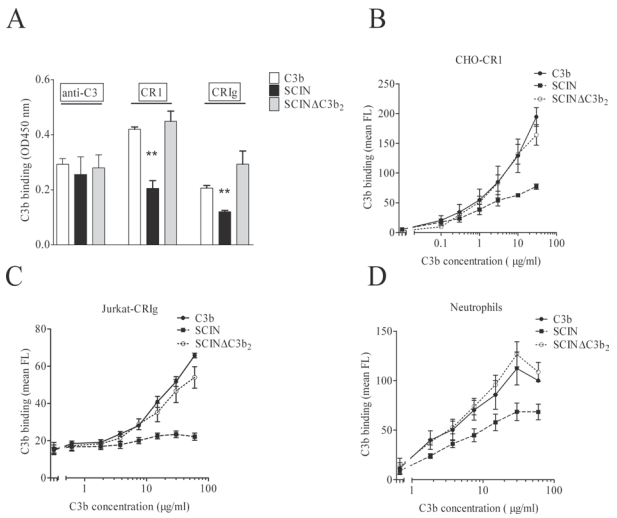

Figure 3: Convertase dimerization results in impaired recognition of C3b by CR1 and CRIg

(a) Binding of soluble C3b or SCIN-convertases to microtiter plates coated with anti-C3c, CR1, or CRIg. Data represent the mean ± SEM of three separate experiments, ** P< 0.01. (b,c,d) Binding of soluble C3b, dimeric or monomeric SCIN-convertases to cell lines expressing CR1 (b) and CRIg (c) or freshly isolated human neutrophils (d). Data represent the mean ± SEM of three separate experiments. See supplementary figure 2 for representative histogram.

The amount of C3b molecules present inside the complexes was determined via SDS-PAGE (supplementary figure 1). Subsequently, the generated fluid-phase convertases were incubated with fH and fI, where after iC3b formation was analyzed. Figure 2d demonstrates that soluble C3b is cleaved by fH and fI into iC3b, as revealed by the disappearance of the C3b α–chain, while C3b molecules incorporated in the dimeric or monomeric SCIN-convertases are not. Thus, dimerization of convertases by SCIN is important for phagocytosis inhibition; however this cannot be attributed to differences in C3b deposition or iC3b formation.

SCIN-induced dimerization of convertases blocks recognition of C3b by complement receptors

As demonstrated above, differences in phagocytosis inhibition between SCIN and SCINΔC3b$_2$ cannot be explained by differences in C3b and iC3b deposition. To investigate whether the recognition of C3b by complement receptors is impaired by SCIN, we first compared the binding of soluble dimeric and monomeric SCIN-convertases to purified CR1 and CRIg by an ELISA. Dimeric SCIN-convertases showed a lower binding to CR1 and CRIg than the monomeric convertases or soluble C3b (figure 3a). We did not observe a difference in the binding of dimeric and monomeric complexes to an anti-C3 antibody, indicating that equal amounts of C3b molecules were present inside the SCIN-convertases (figure 3a).

Next, we studied the binding of soluble SCIN-convertases to surface-expressed complement receptors. Binding of soluble C3b and SCIN dimeric or monomeric convertases to cells expressing CR1 and CRIg also showed that the binding of dimeric SCIN-convertases to CR1 and CRIg was reduced as compared to soluble C3b and the monomeric convertases (figure 3b-c). Also, the binding of dimeric SCIN-convertases to human neutrophils was reduced as compared to C3b and monomeric convertases (figure 3d).

In conclusion, dimerization of convertases by SCIN leads to impaired binding of C3b to purified and cell-bound complement receptors, explaining the difference between SCIN and SCINΔC3b$_2$ in phagocytosis inhibition.

Convertase dimerization occurs on bacterial surfaces and enhances convertase stability

CR1 and CRIg bind less well to soluble dimeric SCIN-convertases than to monomeric convertases. To study whether dimerization of convertases by SCIN occurs on the bacterial surface, bacteria were incubated with human serum in the presence or absence of SCIN or SCINΔC3b$_2$, and C3b deposition or CR1 recognition was measured. SCIN and SCINΔC3b$_2$ equally inhibit C3b deposition (data not shown), but the binding of purified CR1 to bacteria opsonized in the presence of SCIN was less than to bacteria opsonized in the presence of SCINΔC3b$_2$ (figure 4a). This indicates that dimeric convertases are formed on the bacterial surface hiding bacteria for CR1 recognition.

We have previously proposed that the dimeric nature of SCIN-convertases could also contribute to the stability of the surface-bound complex[21]. Although we demonstrate here that stabilization of dimeric versus monomeric convertases during fluid phase conditions does not differ, we investigated stabilization properties on the bacterial surface as well. Bacteria were first incubated with human serum, after which Bb stabilization was measured. Figure 4b-c show that SCIN stabilizes convertases with a half-life time of 4 hours at 37°C on the bacterial surface as compared to a half-life time of 2 hours for the monomeric variant.

Dimerization of convertases by SCIN occurs on the bacterial surface leading to impaired recognition by CR1 and prolonged stabilization of convertases.

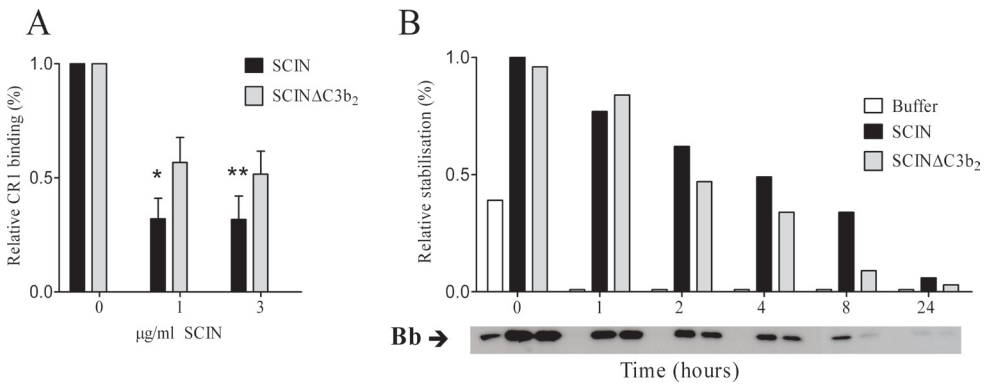

Figure 4: Convertase dimerization occurs on the bacterial surface and enhances stabilization
(a) CR1 binding to *S. aureus* strain Wood 46 opsonized with normal human serum in the presence of SCIN or SCINΔC3b$_2$. Data represent relative CR1 binding (defined as mean fluorescence of sample/ mean fluorescence of control (0 µg/ml protein)) of three separate experiments ± SEM, * P<0.05, ** P<0.01. (b) Bb stabilization on *S. aureus* in the presence of SCIN or SCINΔC3b$_2$. Below, immunoblot of surface-bound Bb. Above, relative convertase stabilization as quantified using Quantity one (Bio-rad). The values are presented as the percentage of Bb stabilization where SCIN at T=0 is 100%. Figure b and c are representative figures of three separate experiments.

DISCUSSION

S. aureus secretes several complement evasion molecules that enable bacterial survival in the human host. These molecules all have unique functional properties and block the complement cascade at different steps[18,26]. For instance, staphylokinase activates human plasminogen at the bacterial surface, thereby removing C3b, which results in the inhibition of phagocytosis[27]. The secreted proteins Extracellular fibrinogen binding protein (Efb) and Extracellular complement binding protein (Ecb) target C3b-containing convertases thereby inhibiting C3b deposition via the AP and C5a generation both *in vitro* and *in vivo*[28]. Next to that, staphylococcal superantigen-like (SSL) 7 directly binds C5[29] which leads to the inhibition of C5a formation and neutrophil migration *in vitro* and *in vivo* (Unpublished data, Bestebroer *et al*). SCIN inhibits C3 convertases of the CP, LP, and AP, thus affects the complement system at a very early stage, and thereby not only inhibits C3b deposition but also phagocytosis and C5a generation. The interaction between SCIN and C3 convertases was shown to be highly human specific, which limits studies in animal models. Nevertheless, SCIN is found in 90% of clinical *S. aureus* strains and is expressed *in vivo*[20]. This and its location on a human-specific bacteriophage[30] implicate a critical role for SCIN in the pathogenesis of *S. aureus* infections in humans.

The co-structure of the AP C3 convertase and SCIN revealed a convertase dimerization site in SCIN that seemed unimportant for blocking convertase activity in fluid phase[21]. It was unclear whether dimerization of AP convertases by SCIN was a fluid phase phenomenon. This paper shows that convertase dimerization also occurs on bacterial surfaces and that it is critical for the immune evasion properties of SCIN. We observe major differences in the anti-phagocytic activity of SCIN and its dimerization mutant. Figure 5a represents a surface representation of the C3b molecule with highlighted complement receptor binding sites[8]. A recent co-structure of CRIg and C3b revealed that CRIg binds the back of C3b[16]. Since this site is close to the C3b:C3b interface found in the dimeric SCIN-convertases, it is likely that sterical hindrance prevents CRIg from binding C3b within the dimerized but not the monomeric complex (Figure 5b)[21]. Binding sites for CR1 are not fully mapped and future studies will be needed to understand the

molecular basis for impaired CR1 binding to the convertase dimer. Recent SPR studies showed that SCIN impairs decay acceleration of the convertase by fH[31]. We find that also the fH- and fI-mediated conversion of C3b to iC3b is blocked in SCIN-inhibited convertases (both monomeric and dimeric). This way, SCIN prevents formation of iC3b on the surface and indirectly provides a way to evade CR3 recognition as well. Dimeric convertases on the surface are more stable which will help the bacteria to evade the complement system for a long period of time. Since CRIg on liver Kupffer cells is critical for clearance of complement-coated pathogens from the blood, this prolonged stability of dimeric convertases enables *S. aureus* to also escape host defenses in later infection stages when bacteria have disseminated from specific tissue sites to the blood[12]. Although it is known that immune evasion molecules excreted by *S. aureus* can interfere with recognition by CR2 on B cells[34,35], SCIN is the first staphylococcal molecule that specifically prevents bacterial recognition by complement receptors on phagocytic cells.

SCIN is a very potent complement inhibitor that evolved in bacteria under constant selective pressure by the human immune system. The interaction between SCIN and the C3 convertase represents a very effective complement evasion strategy that works on several steps in the cascade. Next to reducing both deposition of opsonic C3b on bacteria and release of chemoattractants, we now show that via dimerization of convertases SCIN blocks recognition of deposited C3b by complement receptors. A combination of all these events is probably needed to ensure effective protection from the host immune response. The use of SCIN as a therapeutic in inflammatory diseases is limited due to the presence of preexisting antibodies in humans[19]. However, studying the way bacteria counteract the immune response will be critical in the development of novel anti-inflammatory compounds. Our recent co-structure and the currently presented insights support the development of SCIN derivatives or analogs that effectively modulate both complement activation and phagocytosis.

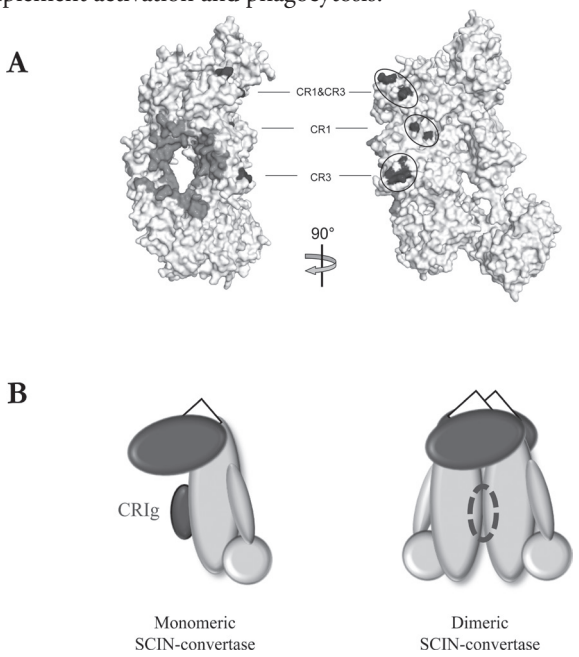

Figure 5: Surface representation of C3b with highlighted complement receptor binding sites
(a) CRIg interfaces (light grey) are based on a co-structure, while CR1 and CR3 binding sites (dark grey) were determined by mutational analyses[14-15; 32-33]. (b) Schematic representation of CRIg binding to the monomeric SCIN-convertase (left). The formation of dimerized C3b molecules within the dimeric SCIN-convertase likely prevents binding of CRIg to C3b.

Acknowledgments

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Chapter 3

Staphylococcal complement evasion by various convertase-blocking molecules

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ABSTRACT

In order to combat the human immune response, bacteria should be able to divert the effectiveness of the complement system. Here we identify four potent complement inhibitors in *Staphylococcus aureus* that are part of a new immune evasion cluster. Two are homologs of the C3 convertase modulator Staphylococcal Complement INhibitor (SCIN) and function in a similar way as SCIN. Extracellular fibrinogen binding protein (Efb) and its homolog Extracellular complement binding protein (Ecb) are identified as potent complement evasion molecules and their inhibitory mechanism was pinpointed to blocking C3b-containing convertases: the alternative pathway C3 convertase C3bBb and the C5 convertases C4b2aC3b and $C3b_2Bb$. The potency of Efb and Ecb to block C5 convertase activity was evidenced by their ability to block C5a generation and C5a-mediated neutrophil activation *in vitro*. Further, Ecb blocks C5a-dependent neutrophil recruitment into the peritoneal cavity in a murine model of immune complex peritonitis. The strong anti-inflammatory properties of these novel *S. aureus*-derived convertase inhibitors make these compounds interesting drug candidates for complement-mediated diseases.

INTRODUCTION

The complement system is essential for an effective immune response against invading pathogens. Conserved microbial sugars or microbe-bound antibodies initiate a cascade of protein-protein interactions and proteolytic steps which result in formation of target-bound convertases that cleave C3 and C5 into biologically important complement fragments[1,2]. The small fragments C3a and C5a serve as potent pro-inflammatory mediators that attract phagocytes to the site of infection and activate them. Deposition of large C3 cleavage products (C3b and iC3b) on microbial surfaces greatly enhances bacterial uptake by phagocytic cells[3,4]. Furthermore, complement can directly kill gram-negative bacteria via formation of a membrane attack complex (MAC), comprised of C5b, C6, C7, C8 and multiple C9, that inserts into target membranes and promotes osmotic lysis[5]. Next to its beneficial role in host defense, complement also promotes many unwanted inflammatory reactions in pathological conditions such as autoimmune diseases[6], allergies[7], allograft/xenograft rejection[8] and systemic inflammation during sepsis[9].

Complement activation occurs via three different recognition pathways that converge at the level of C3 by forming C3-cleaving proteases, the C3 convertases[10]. Activation of the classical (CP) and lectin pathway (LP) results in the cleavage of C4 and C2 by C1s or Mannose Binding Lectin (MBL)-associated Serine Protease-2 (MASP-2) generating a C4b2a complex, the C3 convertase of the CP/LP[11,12]. The alternative pathway (AP) is initiated by formation of either spontaneously hydrolyzed C3 ($C3_{H2O}$) in fluid-phase or surface-bound C3b deposited by the CP/LP. Generated C3b can form a complex with factor B that is activated by factor D to generate the C3bBb complex, the C3 convertase of the AP[13].

The C3 convertases (C4b2a and C3bBb) are of major biological significance as they mediate the release of C3a and the deposition of large numbers of C3b molecules onto bacterial surfaces. C3 convertases consist of a covalently surface-bound noncatalytic subunit (C4b or C3b) which is in complex with the catalytic subunit (C2a or Bb). Due to the rapid and irreversible dissociation of the catalytic subunits, convertases only have a short life time of 1-2 minutes. C3 convertases also function as precursors of C5 convertases[11,14,15]. In response to the deposition of high C3b concentrations on target surfaces, C3 convertases switch to C5 convertases to cleave C5, initiating the release of C5a and the formation of the cytolytic MAC[16-18]. During this process, assembly of one C3b molecule on the C3 convertase results in the formation of C4b-C3b or C3b-C3b dimers that have high affinities for C5.

In recent years it has become evident that bacteria have evolved sophisticated strategies to escape complement-mediated immune responses. *S. aureus* secretes several complement modulators to target different steps in the complement cascade[19,20]. Phagocyte activation by C5a is effectively blocked by the chemotaxis inhibitory protein of *S. aureus* (CHIPS) that binds the C5a receptor (C5aR), as well as the formylated peptide receptor (FPR)[21]. The recently described Staphylococcal Complement INhibitor (SCIN) interferes with all complement activation pathways by blocking C3 convertases[22-24]. The CHIPS and SCIN genes are part of the first immune evasion cluster (IEC-1) in *S. aureus,* a bacteriophage-localized element that also encodes Staphylokinase and Staphylococcal Enterotoxin A[25]. Although CHIPS and SCIN are promising molecules to target complement diseases, their strict specificity for human complement proteins has limited activity studies *in vivo*. Nevertheless, their importance as staphylococcal immune modulators prompted us to seek homologous proteins in *S. aureus*. This led to the identification of a CHIPS homolog (28% amino acid homology) that blocks FPR-like 1 (FPRL1) receptor signaling: FPRL1-Inhibitory Protein (FLIPr)[26]. A 73% homolog of FLIPr (FLIPr-like) is also involved in modulation of formylated peptide receptors (personal communication).

In this study we identify two SCIN homologs that have similar complement inhibitory properties as SCIN. Intriguingly, the genomic location of both CHIPS and SCIN homologs reveals a novel second immune evasion cluster in *S. aureus*, IEC-2. Extracellular Fibrinogen Binding protein (Efb) and its homolog Extracellular complement binding protein (Ecb), both located on IEC-2, are now identified as potent complement inhibitors acting via a novel convertase-inhibitory mechanism. Since complement inhibition by Efb and Ecb is not human-specific, we could for the first time demonstrate the *in vivo* potency of an *S. aureus* derived complement inhibitor.

MATERIALS AND METHODS

Recombinant production of *S. aureus* proteins

To enable protein expression with a cleavable N-terminal Histidine (His) tag, genes (without signal sequences) were cloned into the pRSETB vector (Invitrogen, Paisley United Kingdom) (Primers listed in Table I, Supplementary Materials). Cloning of SCIN homologs was performed as described[22]. Efb, Efb-C and Ecb were cloned by overlap extension PCR. In the first PCR reaction a small region of pRSETB was amplified using XbaI Forward and Enterokinase (EK) cleavage site Reverse (EK-Reverse, Suppl. Table I) primers. The EK-Reverse primers contain a 3'-overhang on the Efb genes. In a second PCR, *efb* and *ecb* were amplified from chromosomal *S. aureus* DNA using EK-Forward primers (to provide a 5'-overhang on the EK site) and EcoRI-Reverse primers (providing a 3'-EcoRI cleavage site). Both PCR products were mixed and amplified in a third PCR reaction using XbaI Forward and EcoRI-Reverse. PCR products and pRSETB were digested with XbaI and EcoRI before ligation. Transformation and expression were carried out as described[21,22]. Proteins were expressed in *E. coli* and purified from bacterial lysates by Nickel affinity chromatography[21]. The His-tag was removed by EK cleavage and separated by a second column passage. The His-tag of Efb was not removed since EK cleavage resulted in unwanted proteolysis. PCR analyses of clinical *S. aureus* strains was performed as described[25] using the pRSETB cloning primers for *orf-d*, *efb* and *ecb* and specifically designed primers for *scb* and *scc* (designated 'I' in Suppl. Table I).

Phagocytosis and complement activation on *S. aureus*

Laboratory strain *S. aureus* Wood was used for phagocytosis and bacterial complement assays. Phagocytosis was performed using FITC-labeled *S. aureus*, human sera and freshly isolated human neutrophils[22,27]. For C5a analyses, heat-killed *S. aureus* (to prevent fMLP production) was incubated with human sera in RPMI medium for 30 minutes at 37°C. Subsequently, collected supernatants were tested for neutrophil calcium mobilization as described[23]. C5a-specificity of this assay was verified by pre-incubation of neutrophils with the C5a receptor antagonist CHIPS-30[21]. To analyze CD11b and CD62L expression, neutrophils were placed on ice for 10 minutes after which expression of CD11b and CD62 was measured using PE conjugated anti-human CD62L (BD pharmingen) and FITC-labeled anti-human CD11b[27] and flow cytometry. C3b deposition was performed by incubation of *S. aureus* with 10% human sera in HBS^{++} (Hepes-buffered Saline, 20mM Hepes, 140mM NaCl, 5mM $CaCl_2$ and 2.5mM $MgCl_2$) after which surface-bound C3b was detected with FITC-conjugated $(Fab')_2$ anti-human C3 (Protos Immunoresearch, San Francisco). Exclusive activation of the CP/LP was performed in factor D-deficient serum[22]. The AP was analyzed in serum supplemented with MgEGTA. Prior to calcium mobilization, MgEGTA-containing supernatants were supplemented with 12 mM $CaCl_2$. To analyze binding, *S. aureus* was incubated with His-Efb-C or His-Ecb (10 μg/ml) and human sera for 30 minutes at 37°C. His-tagged proteins were detected using mouse-anti His-Tag Antibodies (Qiagen), FITC-labeled goat anti-mouse IgG (DakoCytomation, Denmark) and flow cytometry.

ELISA's

Complement ELISA's were performed as described earlier with minor modifications[28,29]. Deposited C3b and C5b-9 were detected using anti-C3c WM1 (ATCC, Rockville, MD[27]) and anti-C5b-9 (Abcam, UK) antibodies respectively, followed by peroxidase (PO)-conjugated goat anti-mouse IgG (Southern Biotechnology Associates Inc, Birmingham). Antibody distribution in humans was determined as described[20]. To study binding of complement proteins by ELISA[20,23], inhibitor-coated wells (10 µg/ml) were incubated with human C5, C4, fB, fH C3, C3b, C3d (all from Calbiochem) or C3c (kindly provided by Bert Janssen). Binding of complement proteins was detected using monoclonal antibodies against human C3c (WM1), C3d (Quidel, San Diego), C5/C5b (HyCult Biotechnology, The Netherlands), Bb (Quidel), factor H (Quidel) or C4d (Quidel) and secondary PO-conjugated anti-mouse antibodies.

Convertase analyses

Detection of bacterium-bound convertases after opsonization was performed as described earlier for SCIN[22]. To prepare C3b-covered bacteria, *S. aureus* was incubated with 20% human sera for 30 minutes in HBS^{++} followed by a 30 minute-incubation in PBS at 37°C to dissociate surface-bound C2a/Bb[22]. To create purified convertases on the bacterial surface, C3b-covered bacteria were incubated with fB (40 µg/ml), fD (1µg/ml) and properdin (4 µg/ml) (all from Calbiochem) for 30 minutes at 37°C in HBS^{++}. To analyze stability, convertases were incubated with Efb-C, Ecb or fH (20µg/ml, Calbiochem) for 30 minutes at 37°C in HBS^{++}. Surface-bound Bb was detected by flow cytometry[22]. Cleavage of C3 and C5 by surface-bound convertases was studied on zymosan. Convertases were created by incubating zymosan particles with serum in PBS supplemented with 2mM $NiCl_2$ (to increase convertase stability) for 30 minutes at 37°C. Washed zymosan particles were subsequently incubated with Efb-C, Ecb (10 µg/ml) or PBS for 5 minutes at 37°C before human C5 or C3 (4 µg/ml, Calbiochem) were added for 1 hour at 37°C. For C3 activation, zymosan was opsonized in C2-deficient serum (Sigma) to exclusively form AP convertases. C5 cleavage was assessed using particles opsonized in normal serum. Formation of C5a or C3a was measured by analyzing supernatants in a calcium mobilization assay. Alternative pathway hemolytic assays were performed as described earlier[22].

Preparation of mouse EGTA plasma

Blood was drawn from C57BL6 (n=10) and A/J mice (n=10) by cardiac puncture in syringes containing EGTA (0.2M; Sigma) as anticoagulant. Cells were pelleted by centrifugation in a swing-out rotor at 1400 x g for 10 min at 4°C.

Zymosan-induced complement activation

EGTA plasma (2.5 ml each) from either C57BL6 or A/J mice was heated to 37°C for 30 min in the presence or the absence of 50µg/ml of Ecb. The plasma was then treated with 1.335 mg PL-2-mercaptomethyl-3-guanidinoethylthio-propanoic acid (MERGEPTA; Calbiochem), 47µl of 4M 6-aminohexanoic acid (EACA; Fluka), 1 ml of washed Zymosan A and 187.5µl of 0.2 M $MgCl_2$ at 37°C for 1 hr to activate the alternative pathway of complement and to block serum carboxypeptidase N. The activated plasma was then centrifuged at 20,000 x g for 1h at 4°C to precipitate large plasma proteins. The supernatant, containing the anaphylatoxins, was stored at -80°C.

Neutrophil chemotaxis

To obtain bone marrow (BM)-derived neutrophils, femurs, tibias and humeri of Balb/c mice were flushed with sterile PBS. The migration of BM-derived neutrophils was determined using a 48 well micro-chemotaxis chamber (Neuro Probe) with polycarbonate filters (pore size 3µm). Twofold serial dilutions (1:2-1:64) of Ecb-treated and untreated zymosan-activated plasma from C57BL6 and A/J mice were generated using 2% BSA-GBSS (Sigma) as diluent. Thirty µl of the diluted plasma was added to the lower well to act as a chemoattractant to 50 µl neutrophils (10^7/ml suspended in 2% BSA-GBSS buffer) which were added to the upper wells. Cells were allowed to migrate for 30 min at 37°C. Membranes were separated, fixed with methanol, and stained with Diff-Quick (BaxterDade). Numbers of neutrophils (per μm^2) migrating

beyond the lower surface of the membrane were determined microscopically (≥20 high power fields; 32x magnification).

Peritoneal Arthus reaction

Balb/c mice were intravenously (i.v.) injected with 100µl of OVA (20 mg/kg body weight, Sigma), immediately followed by intraperitoneal (i.p.) injection of 200 µl rabbit anti-OVA IgG (800µg/mouse, ICN) in sterile PBS. In inhibition experiments, 100 µl of Ecb was administered i.v. and i.p. (600µg/ml) 30 minutes prior to initiation of the Arthus reaction. Further, Ecb was administered i.v. and i.p. using the same concentrations in the absence of the Arthus reaction (as control). Mice of different treatment groups were sacrificed 6 hr after onset of the peritoneal Arthus reaction. The peritoneal cavity was lavaged with 6 ml of ice-cold PBS / 0.1% BSA. Peritoneal cells were washed once with PBS and the cell number was adjusted to $5x10^5$/ml. Fifty µl of this cell suspension were used to prepare cytospin slides, which were stained with Diff Quick (Baxter Dade). Neutrophil numbers (per mm^2) were calculated from ≥20 different microscopic fields. Animal care was provided in accordance with National Institutes of Health guidelines. Animal studies were approved by either the Bezirksregierung Hannover (Hannover, Germany) or the Cincinnati Children's Hospital Medical Center (Cincinnati, OH) institutional animal care and use committee.

Accession numbers

SCIN-B (NP_374275, NP_371683, YP_186032, YP_493754, YP_499659, YP_416506, YP_043217, NP_645858, YP_040544), SCIN-C (YP_040544), ORF-D (YP_039686), Ecb (NP_374271, NP_371679, YP_186027, YP_493750, YP_499654, YP_416502, YP_043213, NP_645854, YP_040541), Efb (NP_374274, NP_371682, YP_186031, YP_493753, YP_499658, YP_416505, YP_043216, NP_645857, YP_040543).

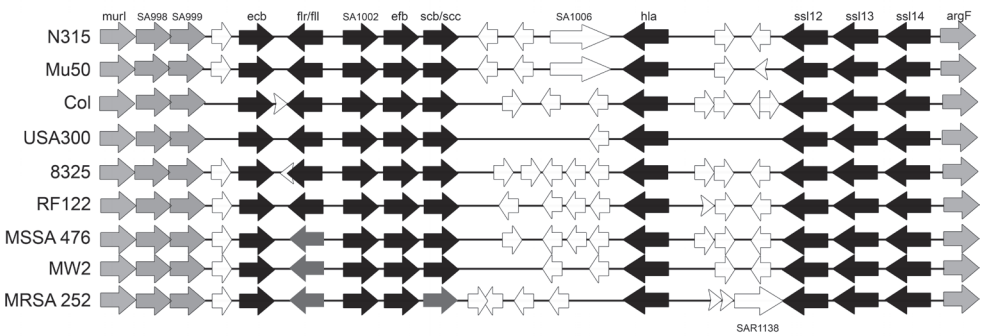

Figure 1: A new Immune Evasion Cluster in *S. aureus*

Graphic representation of the novel Immune Evasion Cluster 2 (IEC-2) in sequenced *S. aureus* strains. Black arrows indicate known or putative immune evasion molecules: Ecb (*ecb*), FLIPr (*flr*), FLIPr-like (*fll*), Efb (*efb*), SCIN-B (*scb*), SCIN-C (*scc*), alpha-haemolysin (*hla*), SSL12 (*ssl12*), SSL13 (*ssl13*) and SSL14 (*ssl14*). Genes with unknown functions are named according to their locus number in *S. aureus* N315. All strains carry either *flr* (black) or *fll* (dark grey) and *scb* (black) or *scc* (dark grey). The household genes (light grey), *murI* (glutamate racemase), *SA998*, *SA999* and *argF* (ornithine carbamoyltransferase) form the borders of IEC-2. White arrows delineate ORFs similar to bacteriophage proteins. Three transposases for Insertion Sequences were found: *SA1006* in N315 and Mu50, *SAR1138* in MRSA252. *S. aureus* strain RF122 represents a bovine isolate.

RESULTS

CHIPS and SCIN homologs are part of a new immune evasion cluster

Database analyses revealed that *S. aureus* contains three open reading frames (ORFs) with a high homology to SCIN, while no significant homologs were found in other microorganisms. We identified SCIN-B, SCIN-C and ORF-D sharing 48%, 46% and 33% homology with SCIN respectively (Figure S1a). Surprisingly, we found that CHIPS and SCIN homologs are clustered on the genome of all sequenced S. *aureus* strains (Figure 1). The cluster contains the gene for FLIPr (*flr*) or FLIPr-like (*fll*) in combination with the gene for SCIN-B (*scb*) or SCIN-C (*scc*). The ORF-D gene is outside this cluster, directly upstream of the staphylocoagulase precursor gene. Next to CHIPS and SCIN homologs, we found this cluster to harbor the genes for Efb (*efb*) and a 33% homolog of Efb that we named Ecb (*ecb*). Efb has dual functions since its N-terminus binds fibrinogen while its C-terminal part (Efb-C) binds different C3 molecules[30-33]. The homology between Efb and Ecb is mainly located in the C3-binding domain of Efb (Figure S1b). Both the C3-binding properties of Efb-C and the high homology with Ecb suggest that these molecules may play an important role in staphylococcal complement evasion. Furthermore, we found this cluster to encode a putative outer membrane protein (SA1002), alpha-haemolysin and three exotoxin-like molecules. Since the exotoxins are structural homologs of staphylococcal superantigens they are referred to as Staphylococcal Superantigen Like 12 (SSL12), SSL13 and SSL14 (personal communication,[34]). The borders of IEC-2 are represented by the housekeeping genes *murI* (glutamate racemase) and *argF* (ornithine carbamoyltransferase), as well as SA0998 and SA0999 (homologs of housekeeping genes). Of note, the presence of transposases and bacteriophage remnants suggests this cluster has evolved through horizontal gene transfer[35]. In summary, the clustering of several known and potential immune evasion molecules indicates this region represents a novel second immune evasion cluster in *S. aureus* (IEC-2). In this paper we will investigate the immune evasive properties of four molecules on IEC-2: SCIN-B, SCIN-C, Efb and Ecb.

Prevalence of SCIN and Efb homologs in *S. aureus* strains

PCR analyses of eighty-four clinical *S. aureus* strains and six classical lab strains revealed that the genes encoding SCIN-B (*scb*), SCIN-C (*scc*) and ORF-D (*orf-d*) are carried by 47%, 32% and 98% *S. aureus* strains respectively. As observed for sequenced strains, clinical *S. aureus* strains also carry either *scb* or *scc*. The genes encoding Efb (*efb*) and Ecb (*ecb*) were found in 85% and 98% of *S. aureus* strains respectively. All are secreted proteins since they contain a signal peptide and a signal peptide cleavage site.

Innate immune evasion by SCIN homologs, Efb-C and Ecb

Recombinant SCIN homologs, Efb-C and Ecb were tested in several *in vitro* assays that mimic critical steps of complement-mediated immune responses against *S. aureus.* To analyze complement activation at the level of C5a, *S. aureus* was first incubated with human serum in the presence or absence of inhibitors. Collected supernatants were subsequently tested for their capacity to activate neutrophils, a response that is completely C5a-dependent[23]. We observed that SCIN-B and SCIN-C effectively blocked C5a-mediated calcium mobilization while ORF-D had no effect (Figure 2a). C5a responses were inhibited by Efb-C and Ecb as well (Figure 2b). C5a responses were not affected when proteins were added to supernatants after opsonization. As a different read-out for neutrophil activation we analyzed expression of CD11b and CD62L.

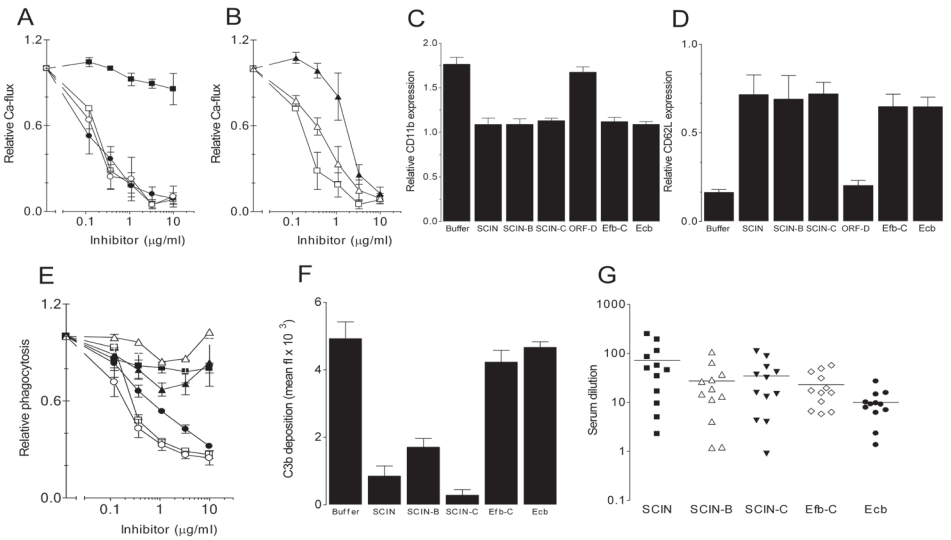

Figure 2: Innate immune evasion by four putative complement inhibitors on IEC-2
(a) SCIN-B and SCIN-C inhibit C5a production. *S. aureus* was incubated with 10% human serum in the presence of 10 μg/ml SCIN (□), SCIN-B (●), SCIN-C (○) or ORF-D (■). C5a formation was measured by using supernatants as a stimulus for calcium mobilization of human neutrophils. SCIN-B and SCIN-C show a dose-dependent inhibition of C5a formation while ORF-D had no effect. (b) Efb-C and Ecb block C5a production during opsonization. Dose-dependent inhibition of C5a-mediated neutrophil activation by Efb-C(▲), Ecb (Δ) and SCIN (□). (c) Supernatants that were activated in the presence of 10 μg/ml SCIN, SCIN-B, SCIN-C Efb-C or Ecb were less potent in up-regulating CD11b expression on neutrophils. (d) Supernatants that were activated in the presence of 10 μg/ml SCIN, SCIN-B, SCIN-C Efb-C or Ecb were less potent in down-regulation of CD62L expression on neutrophils. (e) SCIN (□), SCIN-B (●) and SCIN-C (○) inhibit phagocytosis of *S. aureus* by human neutrophils in 10% human serum. ORF-D (■), Efb-C (▲) and Ecb (Δ) did not affect phagocytosis. (f) SCIN, SCIN-B and SCIN-C inhibit C3b/iC3b deposition on the bacterial surface in 10% human serum while Efb-C and Ecb do not influence opsonization. (g) Antibody titers against SCIN, SCIN-B, SCIN-C, Efb and Ecb in sera of 12 healthy lab volunteers. Mean fl = mean fluorescence. All data represent mean ± SE of three separate experiments.

In concordance with calcium mobilization assays, supernatants generated in the presence of SCIN-B, SCIN-C, Efb-C or Ecb contained less C5a since they could not up- or down-regulate CD11b and CD62L respectively (Figure 2c-d)[36]. Next to C5a formation we studied the effect of SCIN-B, SCIN-C, ORF-D, Efb-C and Ecb on phagocytosis. Neutrophil uptake of FITC-labeled *S. aureus* in the presence of human serum was strongly inhibited by SCIN-B and SCIN-C (Figure 2e). In contrast, Efb-C, Ecb and ORF-D did not affect phagocytosis (Figure 2e), even at different serum concentrations (data not shown). Since effective phagocytosis of bacteria depends on the presence of opsonic C3 fragments[37,38], it was not surprising to find that Efb-C and Ecb did also not inhibit deposition of C3b/iC3b on *S. aureus* (Figure 2f). Only SCIN, SCIN-B and SCIN-C strongly prevented deposition of C3b/iC3b in normal human serum. To study whether SCIN-B, SCIN-C, Efb and Ecb are produced *in vivo*, we analyzed the presence of antibodies against SCIN-B, SCIN-C, Efb-C and Ecb in humans. We found that antibodies capable of recognizing each of the recombinant molecules are present in humans (Figure 2g).

In conclusion, SCIN-B and SCIN-C are efficient complement inhibitors that prevent C5a generation, C3b/iC3b deposition and phagocytosis. ORF-D is not a complement inhibitor and its function remains unknown. In contrast to SCIN molecules, Efb-C and Ecb do not inhibit phagocytosis but strongly interfere with C5a production.

SCIN-B and SCIN-C function similar to SCIN

To gain more insight into the complement inhibitory mechanisms of SCIN-B and SCIN-C we determined their regulatory impact on the different pathways individually[28,29]. ELISA experiments showed that SCIN-B and SCIN-C share the pathway-specificity with SCIN (Figure 3). Both at the level of C3b and C5b-9 formation, SCIN-B and SCIN-C inhibit the CP and LP by 50% (Figure 3a-b,d-e) and the AP by 100% (Figure 3c,f). Of note, SCIN-B appears less potent in blocking CP and LP activation at the level of C3b (Figure 3a-b). Like SCIN, SCIN-B and SCIN-C did not block C4b deposition during CP or LP activation (data not shown). Because stabilization of inherently labile C3 convertases is a hallmark of SCIN activity, we examined convertase-stabilizing capacities of SCIN-B and SCIN-C. Opsonization of *S. aureus* in the presence of SCIN-B or SCIN-C resulted in increased amounts of bacterium-bound C2a and Bb indicating that these molecules stabilize C3 convertases (Figure 3g-h). Collectively, these data show that SCIN-B and SCIN-C inhibit complement by stabilization of C3 convertases which is similar to the inhibitory mechanism of SCIN.

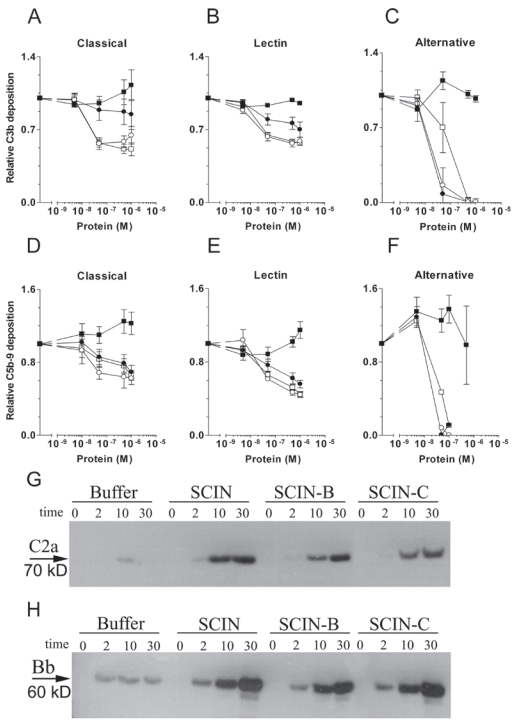

Figure 3: SCIN-B and SCIN-C function similar to SCIN

ELISA experiments showing that SCIN (□), SCIN-B (●) and SCIN-C (○) inhibit C3b deposition following CP (a, 5% serum), LP (b, 5% serum) and AP activation (c, 30% serum). SCIN, SCIN-B and SCIN-C also prevent C5b-9 deposition during CP (d, 5% serum), LP (e, 5% serum) and AP activation (f, 30% serum). ORF-D (■) can not inhibit C3b or C5b-9 deposition in ELISA. Data shown in a-e represent mean ± SE of three separate experiments. (g-h) SCIN, SCIN-B and SCIN-C enhance convertase stability during opsonization of *S. aureus* in 20% human serum. Opsonization was carried out for 0, 2, 10 and 30 minutes. Surface-bound convertases were detected by immunoblotting using anti-C2 (g) or anti-fB (h) antibodies. Blots represent three separate experiments.

Complement inhibitory properties of Efb-C and Ecb

The pathway-specificity of Efb-C and Ecb was also analyzed by ELISA. At the level of C3b deposition we found that Efb-C and Ecb block the AP but not the CP and LP (Figure 4a-c). However, C5b-9 formation was effectively blocked by Efb-C and Ecb in all pathways (Figure 4d-f). In contrast to SCIN, Efb-C and Ecb did not enhance convertase stability during opsonization of *S. aureus* (Figure 4g-h). Since Efb-C binds different C3 molecules via the C3d domain[29], we also studied C3 binding properties of Ecb. Therefore, Ecb was coated to microtiter wells and incubated with C3 or its fragments. Similar to Efb-C, we found that Ecb specifically binds all C3d-containing C3 molecules (C3, C3b, iC3b and C3d) while no binding was observed to C3c which is devoid of C3d (Figure 5a). Binding specificity was demonstrated by a lack of binding to C4, C5, factor B and fibrinogen.

Our findings provide clear evidence that Efb-C and Ecb are potent complement inhibitors that act different from SCIN. By binding to C3d-containing molecules, Efb-C and Ecb can block C3b deposition in the AP, but not in the CP/LP. However, Efb-C and Ecb are potent inhibitors of C5b-9 formation in all complement pathways.

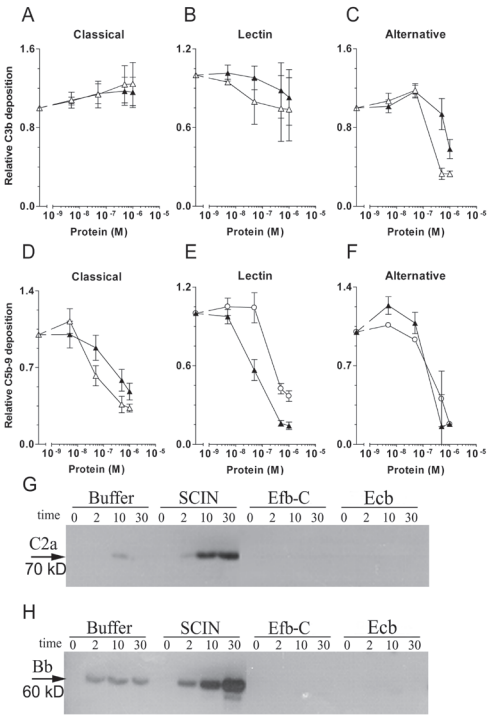

Figure 4: Efb-C and Ecb act different from SCIN

ELISA experiments showing that Efb-C (▲) and Ecb (Δ) do not inhibit C3b deposition following CP (a, 5% serum) or LP (b, 5% serum) activation but strongly block C3b deposition following AP activation (c, 30% serum). Efb-C and Ecb strongly prevent C5b-9 deposition in response to activation of all pathways: CP (d, 5% serum), LP (e, 5% serum) and AP (f, 30% serum). Data shown in a-e are mean ± SE of three separate experiments. (g-h) Efb-C and Ecb do not enhance convertase stability during opsonization of *S. aureus* in 20% human serum. Opsonization was carried out for 0, 2, 10 and 30 minutes. Surface-bound convertases were detected by immunoblotting using anti-C2 (g) or anti-fB (h) antibodies. Blots represent three separate experiments.

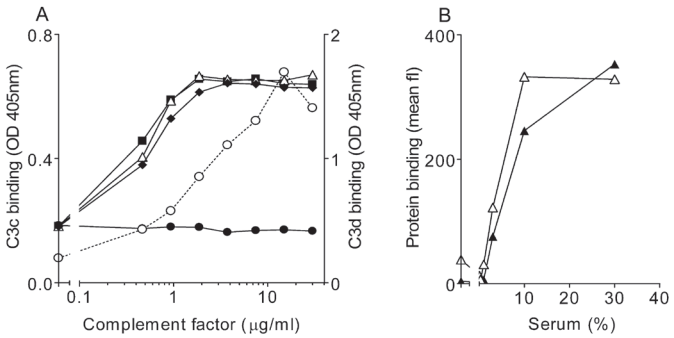

Figure 5: Complement binding properties of Efb-C and Ecb
(a) Ecb binds the C3d domain of C3 (fragments). Ecb (5 μg/ml) was coated to ELISA plates and subsequent binding of C3 (■), iC3b (Δ), C3b (♦), C3c (●) and C3d (○) was determined. Ecb exclusively binds C3d-containing C3 molecules. (b) Efb-C (▲) and Ecb (Δ) bind to *S. aureus* in a serum dependent manner. His-tagged Efb-C and Ecb (both at 10 μg/ml) were incubated with *S. aureus* and human serum for 30 minutes at 37°C and binding was detected using anti-His antibodies and flow cytometry. His-tagged SSL7 served as a negative control. Graphs show one representative figure out of three separate experiments.

Efb-C and Ecb act on the bacterial surface

To assess whether Efb-C and Ecb act on the bacterial surface, we studied binding of His-tagged Efb-C and Ecb to *S. aureus*. The His-tag did not influence complement inhibitory activities as was shown by ELISA (data not shown). We clearly observed a serum dependent binding of His-Efb-C and His-Ecb to *S. aureus* (Figure 5b). The C5-binding protein of *S. aureus*, His-SSL7[19] was used as a negative control and did not bind to the surface. His-tagged Efb-C and Ecb did not bind to bacteria in the presence of heat-inactivated serum, demonstrating a complement-dependent interaction.

Efb-C and Ecb specifically block C3b-containing convertases

Our finding that Efb and Ecb act on the bacterial surface prompted us to study their regulatory impact on C3 and C5 convertases. Therefore *S. aureus* was incubated with human serum and proteolytic activity of C3 convertases was tested by measuring C3b deposition while C5 convertase activity was monitored by testing C5a release in supernatants using neutrophil calcium mobilization assays (Figure 6). To exclusively assess CP and LP convertases, incubations were performed in factor D-deficient serum. AP convertases were analyzed in the presence of Mg-EGTA to chelate calcium ions needed for the CP and LP[22]. We found no inhibition of C3b deposition in the presence of Efb-C and Ecb following CP or LP activation (Figure 6a). In contrast, C5a production was strongly inhibited in response to CP or LP activation (Figure 6c). Importantly, Efb-C and Ecb blocked C3b deposition and C5a-mediated calcium mobilization following AP activation (Figure 6b,d). Collectively, we demonstrate that Efb-C and Ecb do not affect the CP/LP convertase C4b2a, but specifically block C3b-containing convertases, i.e. the AP C3 convertase (C3bBb), the C5 convertases of CP/LP (C4b2aC3b) and AP ($C3b_2Bb$).

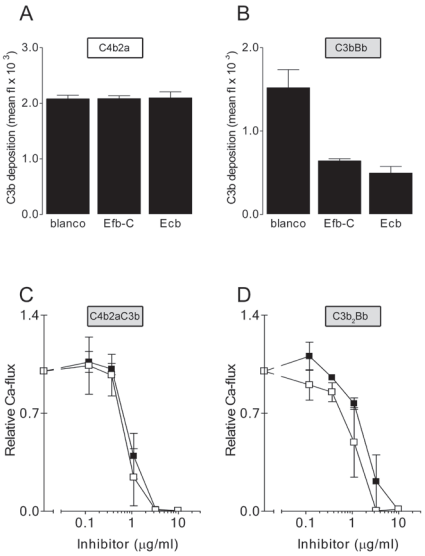

Figure 6: Efb-C and Ecb act on C3b-containing convertases
Efb-C and Ecb specifically block C3b-containing convertases. *S. aureus* was incubated with 10% factor D-depleted serum (to measure CP and LP activation) or 10% human serum in the presence of Mg-EGTA (to measure AP activation). Complement activation was measured at the level of C3b deposition by flow cytometry using anti-C3 antibodies (a,b) or at the level of C5a production by calcium mobilization (c,d). Efb-C (■) and Ecb (□) do not prevent C3b deposition by the CP/LP (a) but inhibit C3b deposition by the AP (b). Formation of C5a was blocked in response to CP and LP activation (c) as well as AP activation (d). Data shown represent mean ± SE of three separate experiments.

Efb-C and Ecb block convertase activity

Since a number of human convertase inhibitors (factor H (fH), Decay Accelerating Factor (DAF)) promote dissociation of convertase complexes, we tested decay accelerating properties of Efb-C and Ecb. Surface-bound AP convertases, created by incubating C3b-coated bacteria with fB, fD and properdin[39], were incubated with Efb-C, Ecb and fH for 30 minutes at 37ºC. Unlike fH, Efb-C and Ecb could not promote dissociation of Bb, indicating they do not act as decay-accelerating molecules (Figure 7a). To investigate whether Efb-C and Ecb affect substrate cleavage by convertases, zymosan-bound convertases were incubated with purified C3 or C5 in the presence or absence of Efb-C and Ecb. Formation of C3a and C5a was measured by neutrophil calcium mobilization. Efb-C and Ecb prevented both C3 cleavage by AP convertases (Figure 7b) and convertases-mediated C5 cleavage (Figure 7c).

These data demonstrate that Efb-C and Ecb prevent convertase cleavage of C3 and C5.

Efb-C and Ecb inhibit complement in various species

The species specificity of SCIN-B, SCIN-C, Efb-C and Ecb was determined in an AP-mediated hemolytic assay where MAC-dependent killing of rabbit erythrocytes is used as a read-out for complement activity. As demonstrated for SCIN, SCIN-B and SCIN-C strongly inhibited AP-mediated hemolysis in human serum (Figure 8a) while no inhibition was observed in mice (Figure 8b) and all other tested species (data not shown). In contrast, we found that Efb-C and

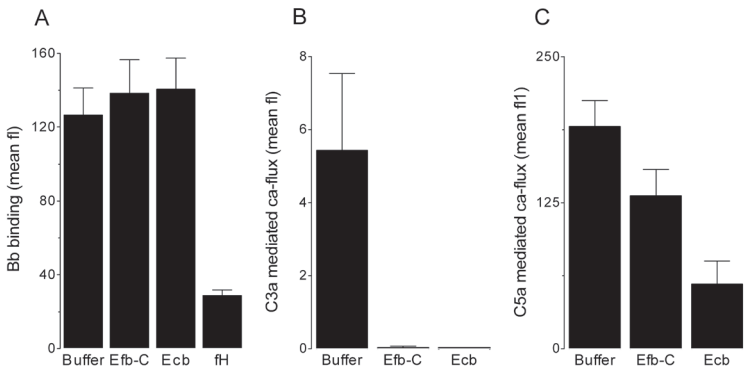

Figure 7: Efb-C and Ecb inhibit C3 and C5 cleavage by convertases
(a) Efb-C and Ecb do not promote decay of convertases. AP convertases were created on the bacterial surface using purified components. Subsequent incubation with inhibitors showed that Efb-C and Ecb do not dissociate convertases like factor H. Surface-bound convertases were detected using anti-Bb antibodies and flow cytometry. (b-c) Efb-C and Ecb inhibit convertase activity. Zymosan-bound convertases were incubated with purified C3 or C5 and generated C3a or C5a was measured in a neutrophil calcium mobilization assay. (b) Efb-C and Ecb inhibit C3 cleavage by AP convertases. (c) Efb-C and Ecb inhibit C5 cleavage by convertases. Mean fl= Mean fluorescence. Data shown represent mean ± SE of three separate experiments.

Ecb not only blocked complement activation in humans, but also in mouse (Figure 8a,b), rat, cow, sheep, goat, guinea pig and dog serum. The fact that Ecb strongly blocks AP activation in mice allowed us for the first time to determine the complement inhibitory properties of an *S. aureus*-derived complement inhibitor *in vivo*.

Ecb strongly blocks IC-mediated inflammation in mice

Immune complexes (ICs) are critical for the pathogenesis of several autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis and glomerulonephritis. In addition to the interaction with IgG Fc receptors (FcγR), ICs also activate the classical and alternative complement pathways and attract various immune cells through the ligation of complement receptors. In the classical experimental model of soluble IC disease, the Arthus reaction, IC activation of local resident cells results in edema, hemorrhage, and neutrophil infiltration. We used the well-established reverse passive Arthus reaction peritonitis model to assess the *in vivo* activity of Ecb[40]. In the absence of inhibitory protein, neutrophils accumulate in the peritoneal cavity within 6 h after IC challenge (Figure 9a). Intraperitoneal and intravenous injection of Ecb prior to IC challenge resulted in complete inhibition of neutrophil migration. Injection of Ecb alone did not induce IC peritonitis. The anti-inflammatory effect of Ecb is similar to deletion of the C5aR or FcγR[40].

To prove that Ecb also prevents C5a generation and C5a-mediated neutrophil migration in mice, zymosan was pre-incubated with EGTA-plasma from C5-sufficient C57BL/6 mice and C5-deficient A/J mice to activate the AP. The plasma was then assessed for its ability to induce migration of bone marrow-derived mouse neutrophils. Strong neutrophil migration occurred in response to C5-sufficient but not C5-deficient plasma (Figure 9b), demonstrating that neutrophil migration is depending on AP-activated C5a. C5-sufficient plasma that had been activated in the presence of Ecb was strongly impaired in its capacity to induce neutrophil migration (Figure 9b). Collectively, these data show that Ecb inhibits AP pathway-induced activation of C5 necessary to induce neutrophil migration both *in vitro* and *in vivo*.

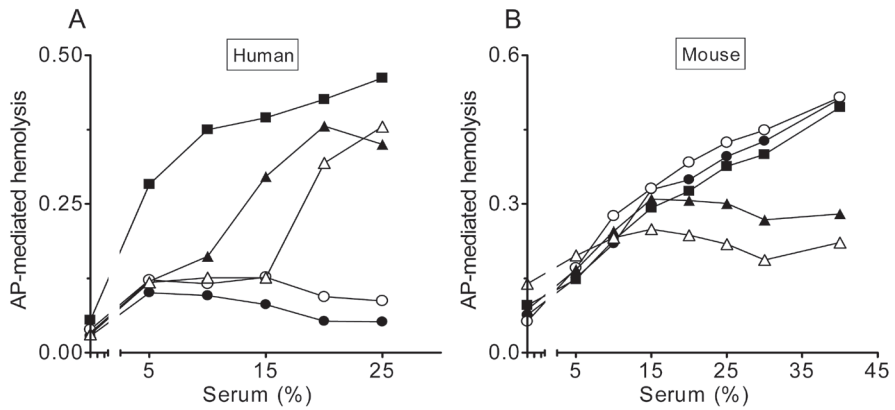

Figure 8: Efb-C and Ecb are not human-specific
(a) SCIN-B, SCIN-C, Efb-C and Ecb inhibit AP-mediated hemolysis of rabbit red blood cells in human serum. SCIN-B (○) and SCIN-C (●) completely block hemolysis at high serum concentrations. Efb-C (▲) and Ecb (Δ) also inhibit AP-mediated hemolysis but are less effective than SCIN-B or SCIN-C (all inhibitors at 10 µg/ml). (b) Efb-C and Ecb inhibit AP-mediated hemolysis in mouse serum. Addition of 10 µg/ml Efb-C (▲) or Ecb (Δ) results in strong inhibition of MAC formation in mouse serum while SCIN-B (○) and SCIN-C (●) had no effect. Graphs show one representative figure out of three separate experiments.

DISCUSSION

The human pathogen *S. aureus* successfully evades innate immune defenses by secretion of numerous small molecules that inhibit critical steps of the immune response. Here we identify SCIN-B, SCIN-C, Efb and Ecb as four new staphylococcal complement modulators that block convertases, the central protease complexes of the complement cascade. Direct convertase modulators are specific for *S. aureus* and provide a very effective way of blocking complement. This paper shows that convertase inhibition is a very powerful mechanism to block inflammatory reactions initiated by complement both *in vitro* and *in vivo*.

The first immune evasion cluster in *S. aureus* (IEC-1) encodes four important immune modulators including SCIN and CHIPS[25]. With the discovery of SCIN and CHIPS homologs we are now confronted with the second immune evasion cluster in *S. aureus*. Six molecules on IEC-2 (FLIPr[26], FLIPr-like (personal communication), SCIN-B, SCIN-C, Efb and Ecb) are now known to have immune evasive properties. The role of the other molecules on IEC-2 is subject for further studies. The immune-modulating properties of SSL12-14 are very likely since the SSLs, structural homologs of superantigens that lack superantigen activity, are believed to modulate various innate immune functions[34]: SSL5 binds P-selectin Glycoprotein Ligand 1 to inhibit neutrophil rolling on epithelial cells[41], SSL7 binds IgA and C5 and blocks IgA-FcR and C5 activation[19]. In contrast to the bacteriophage-located IEC-1, the borders and nature of IEC-2 are less clear. Since IEC-2 contains mobile elements and bacteriophage remnants we believe horizontal gene transfer has played a role in its development. In sharp contrast to IEC-1, IEC-2 is not human-specific as evidenced by its presence in the bovine *S. aureus* strain RF122 and the cross-reactivity of Efb and Ecb in several animal species.

In this paper we highlight two different mechanisms used by *S. aureus* to regulate complement convertases. The SCIN molecules (SCIN, SCIN-B and SCIN-C) specifically block C3 convertases (C4b2a and C3bBb) to prevent C3b/iC3b deposition, phagocytosis and C5a

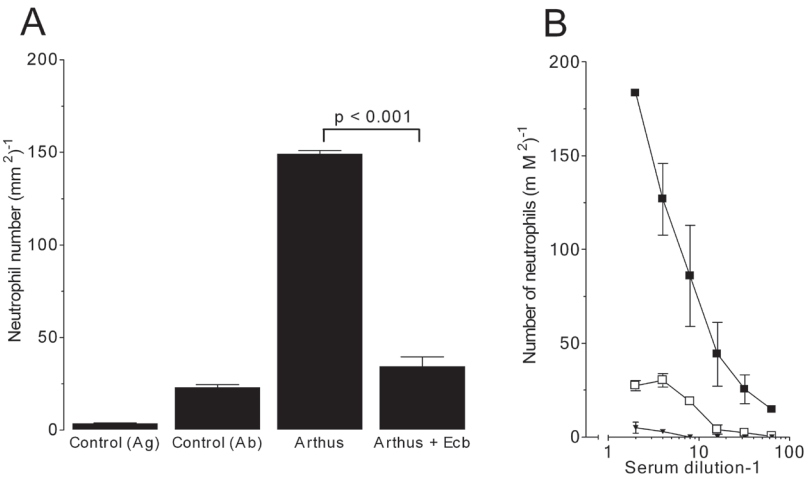

Figure 9: Ecb completely blocks immune complex-induced neutrophil influx *in vivo*
(a) *In vivo* complement inhibition by Ecb was tested in the reverse passive Arthus reaction peritonitis model. Neutrophils accumulate in the peritoneal cavity within 6 h after IC challenge. Intraperitoneal and intravenous injection of Ecb prior to IC challenge resulted in complete inhibition of neutrophil migration. Injection of Ecb alone did not induce IC peritonitis. p<0.001 by ANOVA; n=10/group. (b) Ecb completely blocks C5a-dependent migration of bone-marrow derived mouse neutrophils in vitro. Pre-incubation of zymosan with C5 sufficient (■) but not C5 deficient plasma (▼) results in neutrophil migration. In the presence of 50 µg/ml Ecb, C5a production in C5-sufficient plasma was blocked (□). Data shown represent mean ± SE of three separate experiments.

generation. SCIN does not directly bind C3b, but exclusively binds the activated C3 convertase causing both stabilization and inactivation of this complex[22]. Furthermore, *S. aureus* secretes C3d-binding molecules (Efb and Ecb) that specifically inactivate C3b-containing convertases, i.e. the AP C3 convertase (C3bBb), the C5 convertases of CP/LP (C4b2aC3b) and AP ($C3b_2Bb$). Since inhibition of the AP C3 convertase is not sufficient to block opsonization and phagocytosis in whole serum assays, we believe the major bacterial defense function of Efb-C and Ecb is to block C5a production by C5 convertases and downregulate neutrophil responses. Since previous studies have indicated that Efb-C induces a conformational change in both C3b and C3, it was suggested that Efb-C blocks formation of a functional opsonin[42]. However, our data indicate that Efb-C and Ecb do not directly block C3 cleavage and opsonin formation since C3b deposition by the CP/LP was not affected at physiological inhibitor concentrations. Furthermore, the complement-dependent binding to bacteria demonstrates that Efb-C and Ecb act on the bacterial surface rather than in fluid-phase. The finding that Efb-C and Ecb inhibit the CP/LP C5 convertase (C4b2aC3b) but not the C3 convertase (C4b2a) strongly suggest they bind convertases via C3b. The conformational change in C3b[42] likely affects the activity of the protease complex. We observed that Efb-C and Ecb do not affect the stability of the complex but block cleavage of the substrate (C3 or C5). This and the fact that C3b molecules within C5 convertases provide the additional binding sites for C5[15,17,18,43-45], suggests that Efb-C and Ecb block substrate binding via C3b.

The identification of four novel convertase inhibitors in *S. aureus* illustrates the crucial role of convertase modulation for bacterial survival. It is remarkable that a single bacterium produces five different convertase inhibitors stressing the importance of appropriate complement

activation for *S. aureus* killing. In concert with several additional complement modulators[46], *S. aureus* contains an enormous arsenal of molecules that can block the complement cascade at different steps. These synergistic inhibitory pathways help the bacterium to prevent C5a-mediated neutrophil responses and/or phagocytosis. The fact that all staphylococcal complement modulators are produced *in vivo* suggests they are beneficial for bacterial survival in the human host. The redundancy of this system is reminiscent of the mechanisms used by herpes viruses to escape antigen processing. Similar to *S. aureus*, herpes viruses evade host-mediated immunity by blockade of different steps of the immune response, that is inhibition of different molecules critical for antigen processing[47,48].

The identification of two non-human specific complement modulators allows us for the first time to study the role of complement modulation in bacterial pathogenesis *in vivo*. Ecb strongly prevented the development of neutrophil-mediated inflammation in a murine model of IC-induced peritonitis. Our data suggest that the convertase-inhibitory molecules evolved in *S. aureus* are promising drug candidates for anti-inflammatory therapy although the occurrence of pre-existing antibodies precludes their use in humans in their original form[46]. However, as the active sites of SCIN and Efb-C comprise only minor parts of the molecule[24,42], these molecules are promising lead compounds for development of small non-immunogenic complement inhibitors. The strong anti-inflammatory effect of Ecb *in vivo* illustrates the therapeutic potential of such molecules in severe acute inflammatory disorders.

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Chapter 4

Convertase inhibitory properties of the staphylococcal Extracellular complement binding protein

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ABSTRACT

The human pathogen *Staphylococcus aureus* secretes several complement evasion molecules to combat the human immune response. Extracellular complement binding protein (Ecb) binds to the C3d domain of C3 and thereby blocks C3 convertases of the alternative pathway and C5 convertases via all complement pathways. Inhibition of C5 convertases results in complete inhibition of C5a generation and subsequent neutrophil migration. Here we show that binding of Ecb to the C3d domain of C3b is crucial for inhibition of C5 convertases. Ecb does not interfere with substrate binding to convertases but prevents formation of an active convertase enzyme.

INTRODUCTION

Staphylococcus aureus is a gram-positive bacterium and an important human pathogen that causes community- and hospital-derived infections. Infections can range from mild wound infections and light food-poisoning to severe diseases such as bacteremia and endocarditis[1]. *S. aureus* permanently colonizes 20% of the population and another 60% are intermediate carriers[2]. Bacteria need to be able to survive in the human host to become a pathogen. Therefore, they express adhesion factors, adapt to intracellular milieus or make a thick capsule[3]. Next to these mechanisms, *S. aureus* secretes several small molecules that effectively inhibit the innate immune system and a vast majority appear to have evolved to specifically avoid complement attack[3-5]. Inhibition of the complement system is very important since its activation leads to coverage of bacteria with C3b, resulting in phagocytosis, and formation of C5a which is important for chemotaxis of neutrophils towards the site of infection. The complement system comprises three activation routes: the classical pathway (CP), the lectin pathway (LP) and the alternative pathway (AP). All activation routes converge in the formation of C3 convertases which cleave C3 into C3a and C3b. Next to its importance in phagocytosis, C3b deposition is essential to the downstream formation of C5 convertases which cleave C5 into the strong chemoattractant C5a and C5b, which is essential to the formation of the membrane attack complex[6-10].

Recently, we described the complement inhibitory properties of Extracellular fibrinogen binding protein (Efb) and Extracellular complement binding protein (Ecb)[11] (also known as Ehp[12]). While Efb originally was described as a fibrinogen binding protein[13,14] further study has revealed a modular nature to this molecule: the N-terminus of Efb is responsible for fibrinogen binding but the C-terminus contributes to complement inhibition by binding the C3d domain of C3, C3b, iC3b and C3dg[11,15,16]. Ecb is a homologue of the C-terminal domain of Efb, although it lacks the ability to bind fibrinogen it binds with greater affinity to the C3d domain of C3[11,12]. Efb and Ecb specifically bind to C3b-containing convertases which are the C3 convertase of the AP and the C5 convertases of all complement pathways. They both inhibit C3b deposition via the AP but most importantly they strongly inhibit C5a generation via all pathways, which was demonstrated *in vivo* as well[11]. Although Efb and Ecb completely inhibit C5a generation, the molecular mechanism behind C5 convertase inhibition is not well understood. Since Ecb is the most effective inhibitor of C5a generation and since it exclusively inhibits the complement system as it cannot bind to fibrinogen[11,12] we used this protein and an inactive, site-directed mutant in Ecb to study the mechanism of action on C5 convertases in greater detail.

MATERIALS AND METHODS

Protein expression and purification

Preparation of Ecb (9 kDa) and Ecb N63E/R75E/N82E was described previously. Ecb was expressed with a N-terminal cleavable his-tag to be able to use Ecb with or without His-tag. The His-tag was removed by EK cleavage. Ecb N63E/R75E/N82E was prepared only in the form where its His-tag had been proteolytically removed[11,12]. C3 and fB were purified from freshly isolated human plasma and C3b was generated as described[17]. Human IgG was isolated from pooled serum of healthy volunteers via protein-G purification according to the manufacturer's protocol (GE Healthcare). C1q, C2, C4, C5, properdin and fD were obtained commercially (Calbiochem). Recombinant CRIg-S, the short form of CRIg which binds C3b and iC3b, was obtained from Genentech, Inc.

C5a generation via the CP/LP and the AP

For C5a analyses, heat-killed *S. aureus* (to prevent fMLP production) were incubated with 10% human serum in HBS^{++} (Hepes-buffered Saline, 20mM Hepes, 140mM NaCl, 5mM $CaCl_2$ and 2.5mM $MgCl_2$) for 30 minutes at 37°C in the presence of Ecb or Ecb N63E/R75E/N82E. Subsequently, collected supernatants were tested for the presence of C5a. Freshly isolated neutrophils were incubated with the supernatant and calcium mobilization was measured as described[18]. Exclusive activation of the CP/LP was performed in factor D-deficient serum[19] while activation of the AP was analyzed in human serum in HBS-MgEGTA (Hepes-buffered Saline, 20mM Hepes, 140mM NaCl, 5mM $MgCl_2$ and 10mM EGTA).

CP activation on *S. aureus* with purified proteins

S. aureus strain Wood 46 was cultured overnight on blood agar plates (BD pharmingen) and resuspended in HBS^{++} with 0,1% BSA. To obtain C4b-coated bacteria, 50 µl $1x10^8$/ml bacteria were incubated with IgG (50 µl at 100 µg/ml, 30 minutes at 37ºC), human C1 (50 µl at 30 µg/ml, 40 minutes at 4ºC) and human C4 (100 µl at 30 µg/ml, 40 minutes at 37ºC). Bacteria were washed between every step with HBS^{++} with 0.1% BSA. Subsequently, the bacteria were incubated with 50 µl C2 (5 µg/ml), several concentrations of C3 in the presence or absence of his-Ecb or his-Chemotaxis inhibitory protein of S. aureus (CHIPS) (both at 10 µg/ml) for 30 minutes at 37ºC. C3b deposition was detected using FITC-conjugated $(Fab')_2$ anti-human C3 antibodies (Protos Immunoresearch, San Francisco) and flow cytometry. Binding of his-Ecb and his-CHIPS was detected using mouse-anti His-Tag antibodies (Qiagen) followed by PE-labeled goat anti-mouse IgG (DakoCytomation, Denmark) and flow cytometry. To determine C5a formation by the CP convertase, C4b-coated bacteria were incubated with C2, C3 and C5 in the presence or absence of Ecb (10 µg/ml) for 30 minutes at 37ºC and supernatants were collected by centrifugation. Collected supernatants were tested for calcium mobilization with U937-C5aR cells (as above).

AP activation on *S. aureus* with purified components

Fifty µl *S. aureus* strain Wood 46 ($1x10^8$/ml) were opsonized with 1% human serum in HBS-MgEGTA with 0.1% BSA for 30 minutes at 37ºC. To decay surface-bound convertases, bacteria were washed, taken up in 500 µl PBS and incubated for 1 hour at 37ºC. Pre-opsonized bacteria were then incubated in 50 µl HBS-MgEGTA containing fB (10 µg/ml), fD (1 µg/ml) and several concentrations of C3 in the presence or absence of His-Ecb or His-CHIPS (both at 10 µg/ml) for 30 minutes at 37ºC. C3b deposition was detected by FITC-conjugated $(Fab')_2$ anti-human C3 antibodies. To investigate C5a generation, pre-opsonized bacteria were incubated with 50 µl HBS-MgEGTA containing fB (10 µg/ml), fD (1 µg/ml), C3 (25 µg/ml) and several concentrations of C5 in the presence or absence of Ecb (10 µg/ml) for 30 minutes at 37ºC. Then, supernatants were collected by centrifugation and were tested for C5a calcium mobilization with U937-C5aR cells.

C5 binding ELISAs

Binding of $(C3b)_2$ to C5 was studied as described previously[20] with minor modifications. Microtiter plates were coated with 3 µg/ml anti-C5a antibody clone 2077 (Hycult Biotechnology) and blocked with PBS

with 4% BSA and 0.1% Tween. Between every step, plates were washed with PBS containing 0.05% Tween. Subsequently, C5 (30, 10, 3 µg/ml) was added after washing incubation with $(C3b)_2$ followed in the presence of absence of Ecb or Complement Receptor of the ImmunoGlobulin superfamily (CRIg) (both at 10 µg/ml). Binding of $(C3b)_2$ to C5 was detected using PO-labeled anti-C3 antibodies (Protos Immunoresearch). To determine C5 binding to convertases formed on microtiter plates complement was activated as described with minor modifications[21,22]. Microtiter plates were coated with IgM (CP) or LPS (AP) and blocked with PBS containing 4% BSA and 0.1% Tween. Formation of convertases was achieved by addition of human serum for 1 hour at 37ºC followed by another round of serum incubation for again 1 hour at 37ºC. After washing, convertases were decayed for 1 hour at 37ºC. Purified C5 was added for 1 hour at 37°C in the presence or absence of Ecb or CRIg. C5 binding was detected using an anti-C5a antibody clone 2077 followed by peroxidase (PO)-conjugated goat anti-mouse IgG (Southern Biotechnology Associates Inc, Birmingham).

Conversion of fB

Conversion of fB into Bb was analyzed by incubation of 50 µl zymosan (1 mg/ml; Sigma) with 3% human serum in HBS^{++} and 0.1% BSA. To decay surface-bound convertases, zymosan was washed and incubated for 1 hour at 37ºC in PBS. Washed zymosan was resuspended in 50 µl fB (20 µg/ml) and fD (2 µg/ml) with or without Ecb in HBS^{++} for 30 minutes at 37°C. Washed pellets were boiled in SDS sample buffer and surface-bound Bb was analyzed by immunoblotting using anti-fB polyclonal goat antibody (Quidel) and peroxidase (PO)-conjugated donkey anti-goat IgG (Santa Cruz). To study fB stabilization on the surface, opsonized zymosan was incubated with Ecb (10µg/ml) during the decay period of 1 hour and samples were analyzed by western blot right after decay. To analyze fluid phase conversion of fB into Bb, C3b (10µg/ml) was incubated with fB (5µg/ml) and fD (0.5 µg/ml) in the presence or absence of Ecb or Ecb N63E/R75E/N82E (both at 10 µg/ml) in HBS^{++} for 5 minutes at 37°C. Conversion of fB into Bb was analyzed by immunoblotting as described above.

Stabilization of fB via biacore

Interactions between fB and C3b were analyzed by SPR as described previously[23]. C3b (±9000 RUs) was covalently attached to the surface of a CM5 biosensor chip (GE Healthcare) in 10mM NaAC pH 4.5 (using standard amine coupling). These experiments were performed in HBS-P (0.01M Hepes, 0.15M NaCl, 0.005% surfactant P20, pH7.4, GE Healthcare) at a flow rate of 30 µl/ml. First Ecb (10 µg/ml, ± 5 seconds) was injected to bind (±500 RU) then fB (50 µg/ml) was injected across the surface for 2 minutes followed by dissociation of 2 minutes. To study the impact of Ecb on formation of active convertases, we covalently linked C3b (±10000 RU) on a CM5 chip as previously described[24]. In short, 1000 RU of C3b was coupled to a CM5 chip via amine coupling and subsequently, 50 µl of fB (50 µg/ml) and fD (5µg/ml) was injected followed by 100 µl of C3 (100 µg/ml) in Hepes-Ni^{2+} (Hepes buffered saline, 20mM Hepes, 140nM NaCl, 1mM NiCl) at a flow rate of 20 µl/min. These cycles were repeated until the appropriate amount (10000 RU) of C3b was covalently attached to the surface. Ecb was bound to one flow path as described above. Then, 100 µl of fB (50 µg/ml) and fD 5 (µg/ml) was injected. After 600 seconds, C3 (50 µl, 100 µg/ml) was injected across the surface followed by injection of 50 µl 0.1M Citrate, 1M NaCl, 1mM EDTA, pH 5 to remove non-covalently bound C3/C3b.

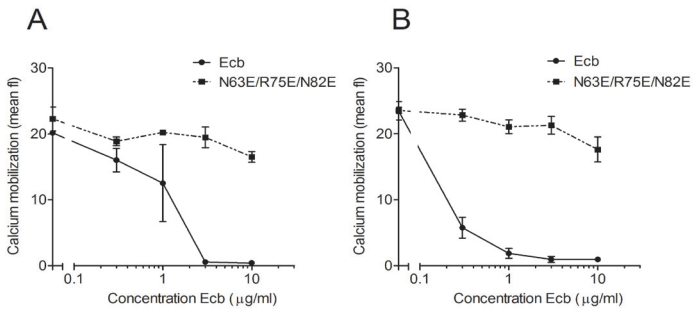

Figure 1: Ecb binding to the C3d domain of C3b is essential for C5 convertase inhibition
C5a generation in the presence of Ecb or its C3d-binding mutant. Bacteria were incubated with human serum and C5a activity in supernatants was analyzed by calcium mobilization of neutrophils. (a) C5a generation via the CP/LP in ΔfD serum. (b) C5a generation via the AP in human serum in the presence of MgEGTA. Both figures represent the mean ± SEM of three separate experiments.

RESULTS

Ecb binding to the C3d domain of C3b is essential for C5 convertase inhibition

C5 convertases are generated by the binding of C3b to pre-existing C3 convertases resulting in the CP/LP C5 convertase (C4b2aC3b) and the AP C5 convertase $C3b_2Bb$[7,8]. We previously observed that Ecb potently blocks C5 convertases. Since Ecb is also known to bind C3b, we wondered whether C3b binding is crucial to C5 convertase inhibition. The binding of Ecb to C3b is well-defined: amino acids N63, R75 and N82 of Ecb bind to the C3d domain of C3b[12]. Subsequently, a C3d binding mutant of Ecb lacking these residues (Ecb N63E/R75E/N82E) does not bind C3b. We used this mutant to study the importance of this interaction in C5 convertase inhibition. We incubated bacteria with human serum in the presence of Ecb or Ecb N63E/R75E/N82E and analyzed C5a generation via calcium mobilization of U937-C5aR. Figure 1 shows that Ecb N63E/R75E/N82E is unable to block C5a generation via the CP/LP (a) and the AP (b). Thus, binding of Ecb to the C3d domain of C3b is essential for inhibition of C5 convertases.

Ecb binds to C3b on the bacterial surface and specifically inhibits C3b-containing convertases

The data above suggest that Ecb inhibits C5 convertases through its interaction with C3b. Recently, we showed that Ecb inhibits C3 convertases of the AP and the C5 convertases of all pathways. Since these experiments were performed in a serum environment we could only speculate how Ecb may block these convertases. To pinpoint the exact mechanism of action, we now studied the inhibitory properties of Ecb in a purified system. Formation of C3 and C5 convertases is a multi-step process and to exactly determine at which stage Ecb binds and blocks convertases we generated surface-bound convertases with purified components and added Ecb at every single stage to look for inhibition of C3b deposition or C5a generation. To generate CP convertases, bacteria were first opsonized with purified human IgG and subsequently incubated with C1. These particles were then incubated with C4 to enable C1-mediated activation of C4 into C4b, which covalently binds to the bacterial surface. The C4b-coated bacteria were incubated with C2 and C3 resulting in formation of C3 convertases (C4b2a) that cleave C3 into

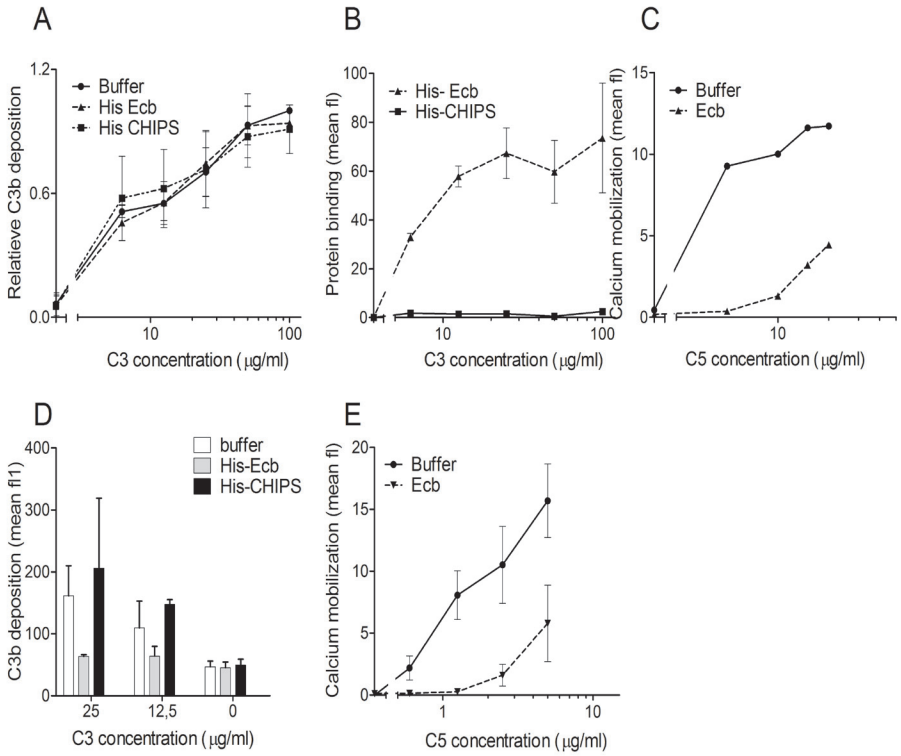

Figure 2: Ecb binds to C3b on the bacterial surface and inhibits C3b-containing convertases
(a) C4b-coated bacteria were opsonized with C2 and C3 in the presence or absence of his-Ecb or his-CHIPS (both at 10μg/ml). (b) Protein binding to the bacterial surface and C3b deposition on the bacterial surface was measured. C3-dependent binding of His-Ecb (10μg/ml). (c) C5a generation via the CP. C4b-coated bacteria were incubated with C2, C3 and C5 in the presence or absence of Ecb. C5a generation was analyzed by calcium mobilization using U937-C5aR cells. (d) C3b deposition via the AP. Pre-opsonized bacteria were incubated with fB, fD and C3 in the presence or absence of Ecb (10 μg/ml). (e) C5a generation via the AP. Opsonized bacteria were incubated with fB, fD, C3 and C5 in the presence of Ecb (10 μg/ml). C5a generation in the supernatant was tested by calcium mobilization. Figure a-b, d-e represent the mean ± SEM of three separate experiments, figure c is a representative of three separate experiments.

C3a and C3b. When C4b-coated particles were incubated with C2 and C3 in the presence of his-Ecb and his-CHIPS (as a negative control[18]) we observed that both proteins failed to inhibit C3b deposition on the bacterial surface (Figure 2a). However, we observed a C3-dependent binding of his-Ecb to the surface indicating that Ecb binds to bacterium-bound C3b molecules during opsonization (Figure 2b). Addition of his-Ecb during incubation of bacteria with IgG, C1, C4 or C2 alone did not result in inhibition of C3b deposition or detectable Ecb binding to the bacterial surface (data not shown). Finally, we studied the effect of Ecb on the CP C5 convertase by incubating C4b-coated particles with C2, C3 and C5 in the presence of Ecb and measuring C5a generation in supernatants by calcium mobilization. Figure 2c shows that Ecb blocks C5a generation by purified CP C5 convertases.

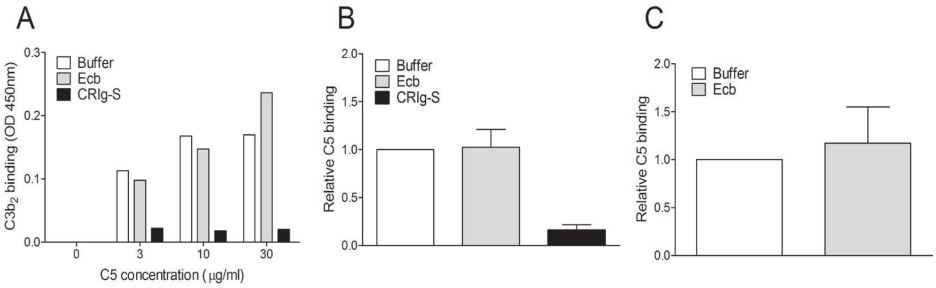

Figure 3: Ecb does not inhibit C5 binding to convertases
(a) $C3b_2$ binding to C5. C5 was captured on anti-C5a coated microtiter plates. Binding of $C3b_2$ to C5 in the presence or absence of Ecb or CRIg-S (both at 10µg/ml) was analyzed. (b) C5 binding to decayed AP convertases in the presence of Ecb or CRIg-S (both at 10 µg/ml). (c) C5 binding to decayed CP convertases in the presence or absence of Ecb (10 µg/ml). Figure a is a representative of three separate experiments while figure b and c represent the mean ± SEM of three experiments.

To generate AP convertases we first opsonized bacteria with human serum. After decay of 1 hour, pre-opsonized bacteria were incubated with fB, fD and C3, which resulted in formation of AP convertases on the bacterial surface (C3bBb and $C3b_2Bb$). When C3b-coated bacteria were incubated with fB, fD and C3 in the presence of Ecb or CHIPS we observed that Ecb, but not CHIPS, blocked C3b deposition via the AP (Figure 2d). This is consistent with recent published data in whole serum where Ecb was shown to inhibit C3b deposition via the AP as well (11). To study the effect of Ecb on AP C5 convertases, C3b-coated particles were incubated with fB, fD, C3 and C5 in the presence or absence of Ecb and C5a generation was measured. Similarly to the CP, we observed that Ecb inhibits C5a generation by purified AP C5 convertases (Figure2e). Similar inhibition was observed when AP convertases were generated in the presence of human properdin, a positive convertase regulator (Supplementary Figure 1).

Together, these studies show that Ecb binds to C3b molecules deposited on the bacterial surface which results in inhibition of C5 convertases of the CP and inhibition of C3 and C5 convertases of the AP.

Ecb does not inhibit the binding of C5 to convertases

Since Ecb inhibits conversion of C5 by C5 convertases, we analyzed whether Ecb can inhibit C5 binding to convertases. The cleavage of C5 by C5 convertases likely occurs in a step-wise process: (a) C5 binds to the C4b-C3b (CP/LP) or C3b-C3b (AP) dimeric component of the convertase[25], (b) the protease component, C2a (CP/LP) or Bb (AP) cleaves C5. Therefore, C5 binding to convertases can be studied in an ELISA format where C5 binds to C4b-C3b generated by decay of convertases on a surface or to preformed $(C3b)_2$[7]. As a control we used CRIg, a complement receptor found on liver Kupffer cells which recognizes opsonized particles via C3b and iC3b[26]. A soluble form of CRIg functions as a complement inhibitor since it binds to C3b and inhibits substrate binding (C3 or C5) to AP convertases[20]. Here, we used CRIg-S a shorter form of CRIg with similar functions. Figure 3a shows that $(C3b)_2$ binding to C5, captured on microtiter plates, is not inhibited by Ecb while CRIg-S does inhibit binding. To investigate C5 binding to naturally formed convertases rather than in a mimicked form, we allowed C5 binding to decayed convertases originated by complement activation on microtiter plates. Again, we observed that

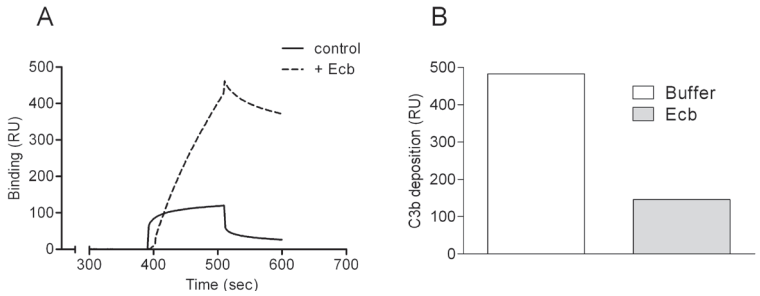

Figure 4: Ecb stabilizes fB binding to C3b and prevents C3bBb formation
(a) FB binding to C3b via biacore. C3b was covalently coupled to the surface of a CM5 biosensor chip where after Ecb or buffer was injected. FB binding was analyzed. (b) C3b deposition on the biacore surface in the presence or absence of Ecb. The figures are representatives of three separate flow paths.

in contrast to CRIg-S, Ecb does not reduce C5 binding to AP convertases (Figure 3b). Similar results were obtained for the CP as Ecb cannot inhibit C5 binding to decayed CP convertases (Figure 3c). All these data provide evidence that Ecb does not interfere with substrate binding to convertases.

Ecb stabilizes fB binding to C3b and prevents C3bBb formation

Since Ecb inhibits C3b-containing convertases without affecting substrate binding, we explored whether Ecb affects the formation of convertases. C3b was covalently coupled to the surface of a CM5 biosensor chip and Ecb was injected to bind ± 500 RU. Recent studies show that the binding between C3b and Ecb is highly stable and can be compared with the affinity for Efb-C with C3b (8.8 nM) (15). Subsequent injection of fB revealed a 4-fold increase of fB binding to C3b chips treated with Ecb (Figure 4a). Next to that, the interaction between fB and C3b appeared more stable in the presence of Ecb. This stabilizing effect was not due to a direct interaction between Ecb and fB since we determined by ELISA that Ecb does not bind fB (Supplementary Figure 2). To investigate whether binding of Ecb to C3b prevented formation of C3bBb on the biacore chip, we first injected fB and fD on a C3b chip treated with or without Ecb. Subsequently, we injected C3 allowing covalent deposition of C3b on the control chip. However, no C3b was deposited on the C3b chip pre-treated with Ecb (Figure 4b). This indicates that Ecb stabilizes fB on C3b and prevents formation of C3bBb.

Ecb prevents fB conversion on the zymosan surface

To investigate whether Ecb can stabilize fB on a natural surface as well, we incubated zymosan with 1% human serum to allow formation of AP convertases and pro-convertases (C3bB). We then studied the decay of surface-bound convertases in the presence or absence of Ecb. We allowed decay of convertases and pro-convertases for 1 hour at 37°C. Although no fB or Bb was detected on the zymosan surface, we found more fB on the surface in the presence of Ecb. This indicates that Ecb stabilizes C3bB on the zymosan surface as well (figure 5a).

To study how Ecb-mediated stabilization of C3bB affects convertase formation and to see whether the C3d binding part of Ecb is essential, we generated fluid-phase AP C3 convertases by incubation of C3b, fB and fD in the presence or absence of Ecb or Ecb N63E/R75E/N82E and

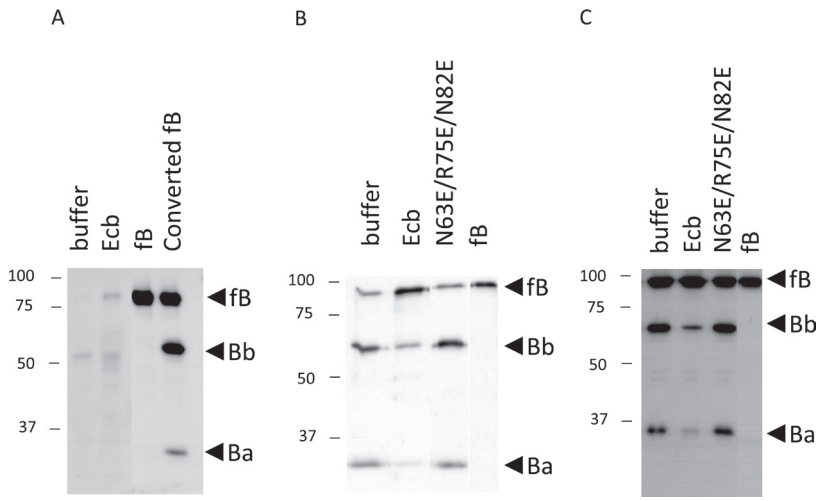

Figure 5: Ecb prevents fB conversion on the zymosan surface
(a) FB stabilization on zymosan in the presence or absence of Ecb (10µg/ml). Zymosan was opsonized with human serum whereupon Ecb was added during decay. FB binding was detected by immunoblotting. (b) Fluid-phase fB conversion. C3b, fB and fD were incubated with or without Ecb or Ecb N63E/R75E/N82E (10 µg/ml) and Bb formation was analyzed by immunoblotting. (c) FB conversion on zymosan. Pre-opsonized zymosan was incubated with fB and fD in the presence or absence of Ecb or Ecb N63E/R75E/N82E(10 µg/ml). Bb formation was analyzed by immunoblotting. All figures are representatives of three separate experiments.

we analyzed Bb formation. Ecb strongly inhibits the formation of Bb in fluid-phase conditions (Figure 5b and supplementary Figure 3) while the C3d binding mutant does not. To analyze the influence of Ecb or the C3d binding mutant on conversion of fB into Bb on the surface, pre-opsonized zymosan was incubated with fB and fD in the presence or absence of Ecb or Ecb N63E/R75E/N82E and Bb formation was analyzed. Figure 5c shows that Ecb, but not the C3d binding mutant, inhibits the formation of Bb on zymosan. These data suggest that Ecb binding to C3b blocks formation of an active convertase enzyme.

DISCUSSION

The covalent deposition of C3b molecules on bacterial surfaces is a key event in the innate immune response[9,10]. Cleavage of C3 into C3b leads to remarkable conformational changes in the C3b molecule, which relocate the 35kD C3d domain and allow covalent binding of its thioester group to the target surface[27,28]. Deposited C3b triggers phagocytosis but also forms an essential part of the convertase enzymes that cleave C3 and C5[7,29,30]. *S. aureus* has developed several ways to survive in the human host. One of these mechanisms is secretion of small molecules that effectively inhibit the complement system[4,5,31].

Among the complement evasion strategies of *S. aureus*, we find a large number of proteins that specifically interact with C3b or C3. The C3 convertase of the AP is a dimeric complex of surface-bound C3b and Bb (Figure 6a). During activation of C3, C3b forms a heterodimer with C3 and thus positions the attached Bb molecule to cleave C3[17]. The AP C3 convertase can be inhibited by several *S. aureus* C3b binding molecules[11,17,32,33]. The Staphylococcal Complement Inhibitor (SCIN) binds C3b in the α'N-terminal tail and domains MG6 and MG7 which are close

to the Bb binding site[17]. Since SCIN also binds to Bb it forms a bridge between C3b and Bb and prevents movement of Bb towards C3 (Figure 6b)[17,34]. Staphylococcal immunoblobulin-binding protein binds to C3[32,35,36] and factor H, and seems to block convertase activity by formation of a tripartite complex of Sbi:FH:C3. The mechanism by which Ecb blocks AP C3 convertases is distinct from SCIN and Sbi. Ecb binds to bacterium-bound C3b during the opsonization process and subsequently stabilizes the C3bB pro-convertase. This stabilized complex cannot be activated into C3bBb. Since the C3d domain of C3b is located far from the fB binding site[28,37] (Figure 6b) a direct interaction between Ecb and fB seems unlikely. Furthermore, Ecb does not seem to bind to other parts of the C3b molecule except to C3d since the C3d-binding mutant is not capable of inhibiting Bb formation. Binding of Ecb to C3d could alter the orientation of the C3b molecule on the bacterial surface. However, conversion of fB into Bb is blocked in fluid-phase as well. Most likely, Ecb binding to C3d results in conformational change in the C3b molecule (as proposed for Efb[15]), causing enhanced binding of fB to C3b. This enhanced binding of fB to C3b could result in inhibition of Bb formation if fD is unable to bind and/or cleave fB in this conformation. Since Efb binds to C3d as well[15] and has similar complement inhibitory functions[11,38], we expect that Efb functions in a similar way.

Next to its effect on the AP C3 convertase, Ecb also blocks the C5 convertases of all pathways. In the CP/LP C5 convertase, C3b incorporates into the pre-existing C3 convertase (C4b2a) to generate the C5 convertase (C3bC4b2a)[8,39-41]. Although C4b is inefficient in capturing C5, the deposited C3b increases the affinity of the enzyme for C5 and thereby alters substrate specificity of the enzyme from C3 into C5[30]. Since Ecb blocks C3bC4b2a but not C4b2a, we previously suggested that Ecb might block C5 binding to C3b[11]. Here, although we demonstrate in more detail that Ecb blocks C5 convertase activity by binding to C3b via its C3d domain, our data show that C5 can still bind to the C5 convertase in the presence of Ecb. Since the structural organization of C5 convertases is presently unknown, it is difficult to speculate how Ecb blocks C5 convertase enzymes of the CP/LP[8,42-44]. Further research is needed to understand how binding to C3b can inhibit the CP/LP C5 convertase. Our data provide

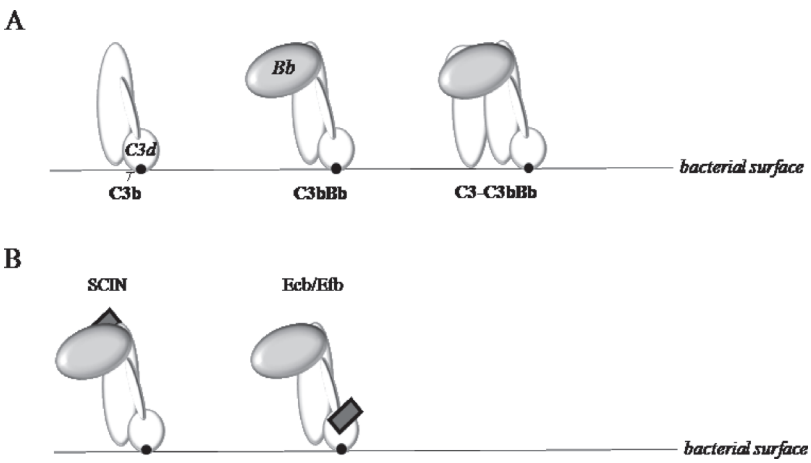

Figure 6: Convertase inhibition by *S. aureus*
(a) Cartoon representation of the covalent attached C3b on the target surface (left), the active C3 convertase (C3bBb, middle) and the binding of C3 to the convertase (right). (b) Cartoon representation of SCIN bound to the AP C3 convertase (left) and Ecb bound to the convertase (right).

compelling evidence that the interaction with C3d is critical for C5 convertase inhibition by Ecb. This indicates that the conformation or spatial orientation of C3b on the surface is important for C5 convertase activity.

Footnote
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Chapter 5

Extracellular complement binding protein inhibits the interaction between C3d and complement receptor 2

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ABSTRACT

Staphylococcus aureus **secretes several complement evasion molecules that enable the bacterium to survive in the human body. A vast majority of these proteins inhibit the complement system. Extracellular fibrinogen binding protein (Efb) and Extracellular complement binding protein (Ecb) specifically bind to the C3d domain of C3, C3b, iC3b and C3dg. Recently, we were able to show their important role in complement inhibition. Both proteins effectively inhibit the formation of C5a. Although it is known that C3d plays an important role in activation of the adaptive immune response, the effect of Efb and Ecb on this response was not yet explored. Here, we show that Ecb effectively inhibits C3d binding to complement receptor 2.**

INTRODUCTION

The human complement system is important for an effective immune response against invading microorganisms. Activation of the complement system results in coverage of the target surface with C3b molecules which is important for phagocytosis. Next, C5 convertases are formed which generate C5a, essential for neutrophil migration towards the side of infection. The complement system also functions as the bridge between innate and adaptive immunity since C3d, a degradation product of C3b, enhances B-cell activation via binding to complement receptor (CR) 2[1-4]. *Staphylococcus aureus* is an important human pathogen causing community- and hospital-derived infections ranging from light food poisoning and uncomplicated wound infections to severe diseases as endocarditis and bacteremia[5]. *S. aureus* secretes several small molecules that effectively inhibit the innate immune system and a vast majority inhibits the complement system[6-9]. Some of these complement inhibitors attack C3, the key molecule in the complement system. Some proteins inhibit C3 cleavage since they inhibit C3 convertases, other proteins bind directly to C3 or its degradation products[10-15]. Extracellular fibrinogen binding protein (Efb) and its C-terminal homologue Extracellular complement binding protein (Ecb) are known for their ability to bind to the C3d domain of C3, C3b, iC3b and C3dg[11,12,14]. Recently, we showed that they specifically inhibit C3b-containing convertases. Ecb prevents formation of the C3 convertase of the alternative pathway (AP) and the C5 convertases of the classical pathway (CP) and the lectin pathway (LP)[14] (Chapter 3).

CR2 is a type 1 transmembrane glycoprotein with a short cytoplasmatic tail and is, depending on alternative splicing, composed of 15 or 16 short consensus repeat domains (SCR). Because of its short cytoplasmatic tail, CR2 needs its co-receptors CD19 and CD81 for signaling. CR2 links the innate and adaptive immune system since binding of an antibody coupled to C3d lowers the threshold for B-cell activation up to a thousand fold[16-19]. C3d binds to the SCR1-2 of CR2 while the antigen binds to the B-cell receptor (BCR). Coupling of CR2 and BCR leads to the coupling between BCR and CD19, which enhances B-cell activation[16,20]. The structures of Efb and Ecb in complex with C3d showed that both proteins bind C3d at a region that is proposed to be involved in the binding of C3d to CR2[11,12,21]. Here, we investigate whether Ecb can prevent C3d binding to CR2.

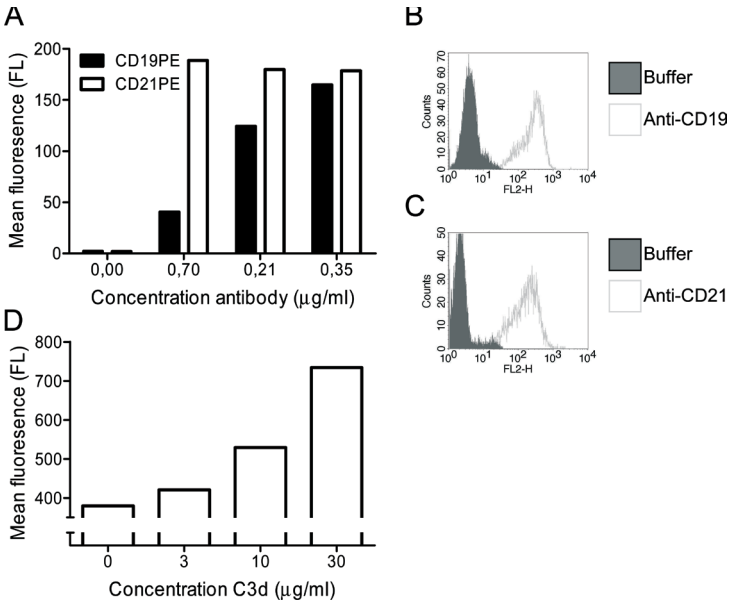

Figure 1: C3d binding to Raji cells
(a) Expression of CD19 and CD21 (CR2) was analyzed on Raji cells by incubating the cells with anti-CD19 or anti-CD21 antibodies. (b) Histogram plots of CD19 expression. (c) Histogram plot of CD21 expression. (d) C3d binding to Raji cells. Raji cells were incubated with C3d where after binding was detected with a biotin-labeled anti-C3d antibody followed by streptavidin-PE. Both graphs are representative graphs of 2 (figure a) or 3 (figure d) separate experiments.

MATERIALS AND METHODS

Proteins and cells

Preparation of Ecb was described previously[14]. Ecb was expressed with a N-terminal cleavable his-tag to be able to use Ecb with or without His-tag. The His-tag was removed by EK cleavage[14]. C3d was obtained commercially (Calbiochem). Raji cells were grown in RPMI 1640 containing 10% FCS and 10 µg/ml gentamicin (Life Technology, Carlbad, CA).

CR2 expression on Raji cells

Raji cells were harvested and washed with RPMI 1640 containing 0.05% human serum albumin (HSA) (RPMI-HSA) (Sanquin). Fifty µl if Raji cell ($5x10^6$) were incubated with PE-conjugated anti-CD21 antibodies (BD Pharmingen) or PE-conjugated anti CD19 antibodies (BD Pharmingen) (both at 0, 0.07, 0.21 or 0.35 µg/ml) for 30 minutes at 4°C. After washing with RPMI-HSA binding was analyzed using flow cytometry.

C3d binding to Raji cells

Raji cells were harvested and washed with RPMI-HSA. Fifty µl cells ($5x10^6$) were incubated with C3d (30, 10, 3 and 0 µg/ml) for 30 minutes at 4°C. Between every step cells were washed with RPMI-HSA. Subsequently, C3d binding was detected using biotinylated anti-C3d antibodies (Quidel, San Diego, CA) followed by PE conjugated streptavidin (R&D systems). Binding was analyzed using flow cytometry. If the C3d binding to Raji cells was tested in the presence of Ecb, 5 µl C3d (30 µg/ml) and 5 µl Ecb (20, 10 or 5 µg/ml) were pre-incubated for 10 minutes at room temperature whereupon 45 µl of Raji cells ($5x10^6$) were added for 30 minutes at 4°C. C3d binding was detected as described above. Data were analyzed by a two-tailed unpaired student's T-test.

C3d binding to human B-cells

Five µl of C3d (25, 12.5, 6.25 and 0 µg/ml) and 5 µl of Ecb (3µg/ml) were pre-incubated for 10 minutes at room temperature after which 45 µl of human peripheral blood mononuclear cells (PBMC's) ($1x10^7$) were added for 30 minutes at 4ºC. After washing with RPMI-HSA, 3 µg/ml anti-C3dg antibodies (Quidel) were added for 30 minutes at 4ºC together with FITC-conjugated goat anti-mouse antibody (DakoCytomation, Denmark). Subsequently, cells were washed again followed by addition of PE labelled anti-CD19 antibody for 30 minutes at 4ºC whereupon C3d binding to CD19 positive B-cells was analyzed by flow cytometry. Data were analyzed by a two-tailed unpaired student's T-test.

RESULTS

C3d binding to Raji cells

To prevent over activation of the complement system, C3b attached to a target surface is degraded by factor I (fI) in the presence of cofactors factor H or CR1. Cleavage of C3b by fI first results in the formation of iC3b, further cleavage results in the formation of C3d on the target surface[22-24]. C3d is an important link between the innate and adaptive immune system since C3d binding to CR2 on B-cells lowers the threshold for B-cell activation up to a thousand fold[16]. To test whether Raji cells, a Burkit lymphoma cell line, could function as a model cell line to test the effect of

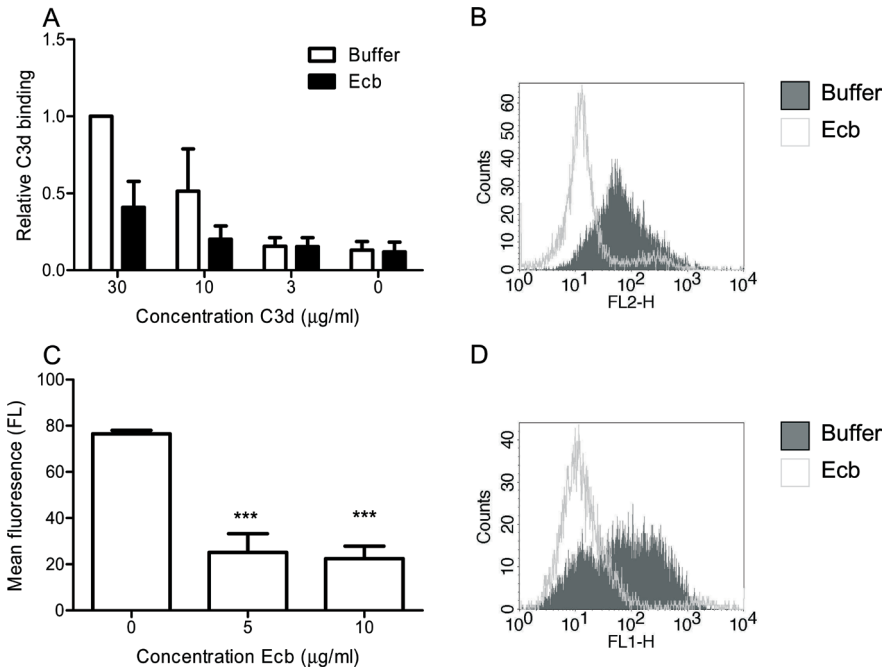

Figure 2: Ecb inhibits C3d binding to Raji cells

(a) C3d binding to Raji cells in the presence of Ecb. C3d was pre-incubated with Ecb (20 µg/ml) where after C3d binding to Raji cells was detected using a biotin labeled anti-C3d antibody followed by streptavadin-PE. Ecb inhibits C3d binding to Raji cells. (b) Histogram plot of C3d (30µg/ml) binding to Raji cells without or with Ecb. (c) C3d binding to Raji cells in the presence of different concentrations of Ecb *** P< 0.001. (d) Histogram plot of the C3d (30 µg/ml) binding to Raji cells in the presence or absence of Ecb (5 µg/ml). Figure a and b represent the mean ± SEM of three different experiments.

Ecb on C3d binding to CR2, we explored its expression of CR2 and its co-receptor CD19. Raji cells were incubated with anti-CD21 (CR2) and anti-CD19 after which binding was analyzed by flow cytometry. Figure 1a-c shows that CD19 and CR2 are expressed on Raji cells. To examine whether Raji cells are able to bind C3d, cells were incubated with several concentration of C3d whereupon C3d binding was analyzed. We observed a dose dependent binding of C3d to Raji cells (Figure 1d).

Thus, Raji cells express CR2 and bind C3d. Therefore, they can function as a model cell line to study the effects of Ecb on C3d binding to CR2.

Ecb inhibits C3d binding to CR2

Raji cells are shown to be a good model to study C3d binding to CR2. Since it is shown that the binding places of Ecb to the C3d molecule are close to the binding site predicted for binding of C3d to CR2[11,21] we studied whether Ecb is capable to inhibit the binding of C3d to CR2. Ecb (20 μg/ml) and several concentrations of C3d were pre-incubated after which C3d binding to CR2 was analyzed showing a reduction of C3d molecules bound to CR2 in the presence of Ecb (Figure 2a-b). To investigate whether lower concentrations of Ecb could inhibit C3d binding as well, we investigated the amount of Ecb needed to inhibit C3d binding and we observed that 5 μg/ml Ecb is sufficient to block this binding (Figure 2c-d). In conclusion, Ecb is a potent inhibitor of C3d binding to CR2 on Raji cells.

Ecb inhibits C3d binding to human B-cells

We already showed that Ecb inhibits C3d binding to Raji cells. To study the effect of Ecb binding to human B-cells in a more natural setting we pre-incubated C3d with Ecb and studied binding of C3d to CD19 positive human B-cells in a mixture of PBMC's. Figure 3 shows that Ecb, similar as already described for Raji cells, inhibits C3d binding to human B-cells as well.

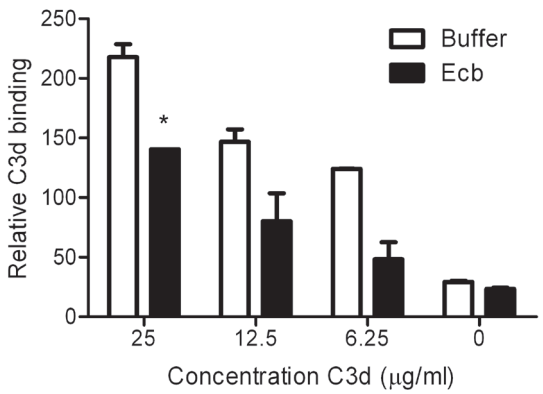

Figure 3: Ecb inhibits C3d binding to human B-cells

C3d binding to CD19 positive human B-cells in the presence or absence of Ecb. C3d and Ecb were pre-incubated an then incubated with human PBMC's. C3d binding was detected using anti-C3d antibody followed by FITC conjugated goat anti-mouse antibody. CD19 positive cells were analyzed for C3d binding. Ecb inhibits binding of C3d to B-cells * $P<0.05$. Figure is the mean ± SEM of two different experiments.

DISCUSSION

S. aureus effectively inhibits the human innate immune system by secretion of several immune evasion molecules[6-8]. Next to that it seems like *S. aureus* inhibits the adaptive immune response as well, which results in a limited formation of antibodies against *S. aureus*. Recovery from an *S. aureus* infections does not result in a protective immune response against subsequent infections[25]. One of the proteins that is known to influence B-cell activation is Staphylococcal protein A (SpA). SpA binds to the Fc-part of IgG molecules thereby inhibiting Fc-receptor mediated phagocytosis by neutrophils[26]. Next to this, SpA is also known for its superantigen activity[27]. SpA binds to B-cells expressing V_H3-family related antibodies. B-cells expressing V_H-3 antibodies are shown to be highly important for the production of antibodies against highly conserved bacterial determinants such as capsular polysaccharides. Binding of SpA and other superantigens to B-cells results in B-cell proliferation, activation, migration and deletion. Although SpA is known to be important for virulence of *S. aureus*, more experimental data need to be obtained to show exactly how superantigens influence the adaptive immune system[27]. Here, we show that *S. aureus* produces other molecules that have the capacity to inhibit the adaptive immune response. Efb and Ecb were known for their capacity to effectively inhibit the innate immune system, but they also bind to C3d, which is a key molecule in the adaptive immune response. Here, we show that Ecb effectively inhibits C3d binding to CR2. It is generally accepted that C3d binding to CR2 lowers the threshold for B-cell activation up to a thousand fold[16] so although we did not show inhibition of B-cell proliferation by Ecb we do think that Ecb inhibits the adaptive immune response. Next to that, synchronous research showed that Efb inhibits the binding of C3d to CR2 as well. Moreover, Efb effectively inhibits B-cell proliferation[28].

It is well established that C3d binding to CR2 enhances B-cell activation but the exact binding place of C3d to CR2 remains unclear[29]. The structure of C3d revealed an acidic pocket as a possible CR2 binding site[30]. Mutations on both sides of this pocket indeed showed complete loss of binding between C3d and CR2[31]. In contrast, the structure between CR2 SCR1-2 and C3d reveals a different binding site on the C3d molecule[32]. This binding place is debatable since the structure of C3 revealed that this binding place is fully exposed in native C3 which is unable to bind to CR2[29]. Our data indicate that the acidic pocket on C3d which is proposed as the binding place of C3d to CR2 is the most likely binding place since Ecb, which binding site to C3d is close to this acidic pocket, effectively inhibits C3d binding to CR2.

Our data shown here are in consensus with simultaneous research by Daniel Ricklin who showed that both Ecb and Efb inhibit C3d binding to CR2[28]. Taken his and our data together we can state that expression of Ecb and Efb by *S. aureus* is very effective to evade the human immune system. The proteins first effectively inhibit C5a generation preventing migration of neutrophils towards to side of infection, next, they inhibit activation of the adaptive immune system preventing C3d binding to CR2.

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Chapter 6

Immune evasion properties of C3b binding proteins

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Manuscript in preparation

ABSTRACT

In order to combat the human immune response, the human pathogen *Staphylococcus aureus* expresses several small complement evasion molecules. Extracellular fibrinogen binding protein (Efb) and its C-terminal homologue Extracellular complement binding protein (Ecb) bind to C3b thereby inhibiting C3b-containing convertases. Next, both proteins are able to block C3d binding to complement receptor 2. Here, we show that both proteins are able to prevent binding of soluble C3b to human neutrophils, CHO-cells expressing complement receptor 1 and Jurkat-cells expressing Complement Receptor of the Immunoglobulin superfamily. Moreover, we show that Efb and Ecb prevent rosette formation of opsonized sheep red blood cells with human neutrophils or CHO-complement receptor 1 cells. In addition we show that Efb is a potent inhibitor of phagocytosis. These studies provide insights into the unique immune evasion strategies employed by *S. aureus*.

INTRODUCTION

The human innate immune system is the first line of defense against invading microorganisms[1]. The innate immune system is composed of a cellular and a non cellular component, together they quickly recognize and kill a diverse range of invading microbes without destroying the host tissue. Invasion of the human body by microbes results in activation of the complement system, which covers the target surface with C3b and is responsible for the formation of C5a. Simultaneously, neutrophils are activated via the complement component C5a and bacterial formylated peptides. Activated neutrophils recognize opsonized microbes via complement receptor (CR)1 and CR3. CR1 (CD35) is a large type 1 transmembrane glycoprotein which is composed of tandemly arranged modules of 60-70 amino acids long known as complement control protein repeats (CCPs)[2]. CR1 specifically recognizes C3b and C4b. CR3 (CD11b/CD18) is a 2 integrin that recognizes iC3b and in addition it acts as an adhesion[3]. CR3 is known as an important phagocytic receptor but also cooperates with CR1, CD14 and Fc RII to trigger phagocytosis of the marked microbe[3].

Staphylococcus aureus is a human pathogen that has found several ways to evade the human immune system[4,5]. Next to secretion of toxins and adhesins[6,7], *S. aureus* has a large arsenal of immune evasion molecules including a large amount of complement inhibitory proteins[8]. Extracellular fibrinogen binding protein (Efb) was first identified as a fibrinogen binding protein[9], but today it is also known as a complement inhibitor. The protein binds to the C3d domain of C3, C3b, iC3b and C3dg thereby effectively inhibiting C5a generation[10,11]. Extracellular complement binding protein (Ecb) is a homologue of the C-terminus of Efb. Ecb lacks to ability to bind fibrinogen but, similar to Efb, binds C3d and effectively inhibits C5a generation[11,12].

Recently, it was shown that both Efb and Ecb inhibit C3d binding to CR2[13(Chapter 5)] but the effects of Efb and Ecb on C3b binding to complement receptors was never studied. Here, we show that Efb and Ecb efficiently inhibit C3b binding to neutrophils. Next, both proteins inhibit rosette-formation and Efb is able to inhibit phagocytosis of C3b-coated sheep red blood cells (SRBC) by neutrophils.

MATERIALS AND METHODS

Protein expression and purification

Preparation of recombinant Efb, Efb-C and Ecb was previously described[11]. Efb-F (the fibrinogen binding part of Efb) was a kind gift of YaPing Ko (Texas A&M University Center, USA). C3 was purified from freshly isolated human plasma and C3b was generated as described[14]. FB was purified as described previously[14]. FD and iC3b were obtained commercially (Calbiochem).

Cell lines and maintenance

A Chinese hamster ovarian (CHO) cell line expressing CR1 (CR1-CHO) and a control R-CHO cell line were obtained from Prof. John Atkinson[15]. Cells were maintained in Ham's F12 medium (Gibco) with 10% FCS, 10 µg/ml gentamycin and 500 µg/ml neomycin (Gibco). A Jurkat cell line expressing Complement Receptor of the ImmunoGlobulin superfamily (CRIg) (Jurkat-huCRIg) and Jurkat cells (negative control)[16] (Genentech, Inc) were maintained in RPMI 1640 medium (Life Technology, Carlsbad) containing 10% FCS, 10 µg/ml gentamycin and 500 µg/ml neomycin.

C3b and iC3b binding to cells

Fifty µl neutrophils, CHO-CR1 cells or Jurkat-CRIg cells (all 5 x10^6/ml) were incubated with 50 µl Efb, Efb-C, Efb-F or Ecb (all at 10 µg/ml) for 30 minutes at 4ºC. After washing with RPMI 1640 containing 0.05% HSA (Sanquin) (RPMI-HSA) cells were incubated with several concentrations of C3b or iC3b for 30 minutes at 4ºC. Subsequently, cells were washed and C3b binding was detected using FITC conjugated anti-C3 antibodies (Protos Immuno Research) and flow cytometry. To study binding of C3b pre-incubated with Efb, Efb-C, Efb-F and Ecb to neutrophils and CHO-cells, twenty-five µl of Efb, Efb-C, Efb-F and Ecb (all at 20 µg/ml) and 25 µl C3b (various concentrations, 60 (for CHO-cells) or 30 (for neutrophils) µg/ml) were pre-incubated for 30 minutes at 4ºC whereupon 50 µl of neutrophils or CHO-CR1 cells (both 5 x 10^6/ml) were added for another 30 minutes at 4ºC. C3b binding was detected after washing as described above. Fifty µl neutrophils were incubated with C3b (30 µg/ml) for 30 minutes at 4ºC after washing with RPMI-HSA, cells were incubated with Efb, Efb-C, Efb-F and Ecb (10 µg/ml) for 30 minutes at 4ºC. Cells were subsequently washed with RPMI-HSA and C3b binding was detected as described above.

C3b binding ELISA

Microtiter plates (Greiner Bio one) were coated overnight at 4ºC with Efb, Efb-C, Efb-F or Ecb (all at 3 µg/ml). Plates were blocked with PBS, 0.1% Tween and 4% BSA for 1 hour at 37ºC. Plates were washed three times between every step with PBS with 0.1% Tween. Soluble C3b (15 µg/ml) was added for 1 hour at 37ºC whereupon C3b binding was detected using PO-conjugated anti-C3 antibodies (1:5000) (Protos Immuno Research).

Rosette formation

SRBC (Alsever) were washed three times with PBS whereupon they were diluted in HBS^{++} (Hepes-buffered Saline, 20mM Hepes, 140mM NaCl, 5mM $CaCl_2$ and 2.5mM $MgCl_2$) containing 0.1% gelatin (HBS^{++}-gelatin) to a 4% suspension (3-4 x 10^8 cells/ml). The SRBC were incubated with poly-anti-sheep-antibody for 10 minutes at room temperature subsequently, SRBC were washed with HBS^{++}-gelatin and cells were concentrated to 1 x 10^9 cells/ml. SRBC were opsonized with C3b by incubated with 7.5% C5 deficient serum (Sigma) for 30 minutes at 37ºC while shaking. After washing with HBS^{++}-gelatin, the SRBC were labeled with the PKH2 green fluorescent Cell linker kit (Sigma) according to the manufactures protocol with some minor modification; 1 x 10^9 SRBC/ml were labeled instead of 1 x 10^7 cells/ml and the protocol was performed at room temperature. Subsequently, neutrophils or CHO-CR1 cells were incubated with Efb or Ecb (5 µg/ml). Next, C3b opsonized SRBC were incubated with Efb or Ecb (5 µg/ml) for 30 minutes at 4ºC. After 30 minutes, 50 µl neutrophils or CHO-cells (5 x 10^6/ml) were incubated with SRBC (2 x 10^8, 1 x 10^8, 5 x 10^7, 1 x 10^7 or 0 /ml). Cells were spun down for 1 minute at 4000 rpm whereupon they were incubated for 30 minutes at 4ºC. Cells were resuspended very carefully and fixated with 100 µl 4% Formaldehyde (Polyscience Inc.). Rosette formation was analyzed by flow cytometry.

Phagocytosis

Phagocytosis was performed as previously described[11] with minor modifications. *S. aureus* strain KV27 was opsonized with plasma (10-5-2.5-0%) for 2 minutes at 37ºC before freshly isolated neutrophils were added for another 15 minutes. Phagocytosis was also performed in 5% human serum supplemented with fibrinogen (200-100-50-0 µg/ml).

RESULTS

Pre-incubation of cells with Efb prevents C3b binding

Fluid phase C3b binds to CR1 on neutrophils and to CRIg, the complement receptor expressed on liver kupffer cells[16,17]. Since we previously showed that Efb and Ecb bind to C3b[11] we wondered whether these C3d binding proteins could inhibit binding of fluid phase C3b to freshly isolated neutrophils. Therefore, neutrophils were pre-incubated with Efb and, after washing, incubated with C3b. Figure 1a shows that Efb inhibits C3b binding to neutrophils. Next, we investigated binding of C3b to CHO-cells pre-incubated with Efb. We observed that C3b binding to CHO-CR1 cells was inhibited in the presence of Efb (Figure 1b). To study whether Efb specifically inhibits C3b binding to CR1 or whether C3b binding to other complement receptors can be blocked as well, we analyzed C3b binding to Jurkat-CRIg cells pre-incubated with or without Efb. Figure 1c shows that Efb inhibits C3b binding to Jurkat-CRIg cells just as effective as observed for neutrophils and CHO-CR1 cells.

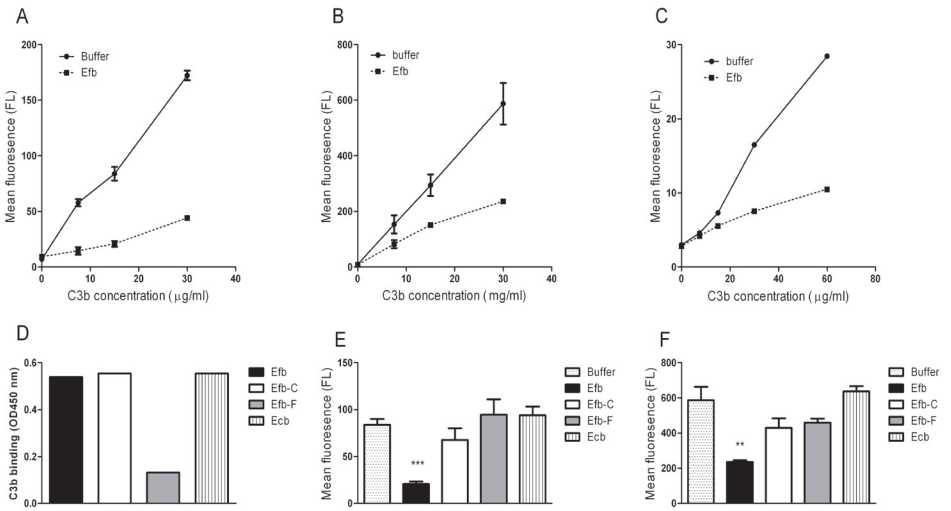

Figure 1: Pre-incubation of cells with Efb inhibits C3b binding

(a) C3b binding to freshly isolated neutrophils pre-incubated with or without Efb (10µg/ml). (b) C3b binding to CHO-CR1 cells pre-incubated with or without Efb (10 µg/ml). (c) C3b binding to Jurkat-CRIg cells pre-incubated with or without Efb (10 µg/ml). (d) C3b binding to immobilized Efb, Efb-C, Efb-F and Ecb (all at 3µg/ml). (e) C3b (15 µg/ml) binding to freshly isolated neutrophils pre-incubated with Efb, Efb-C, Efb-F and Ecb (all at 10 µg/ml). *** P<0.0001 according to the student T-test. (f) C3b (15 µg/ml) binding to CHO-CR1 cells pre-incubated with Efb, Efb-C, Efb-F and Ecb (all at 10 µg/ml). **P<0.001 according to the student T-test. Figure c and d are representative graphs of respectively 2 or 3 separate experiments while figure a-b, e-f represent the mean ± SEM of three separate experiments.

To investigate whether inhibition of C3b binding to neutrophils is a common phenomenon for C3b binding proteins we tested whether Efb-C and Ecb, both able to bind C3b, can inhibit this binding as well. The fibrinogen binding part of Efb (Efb-F) is unable to bind to C3b (Figure 1d) and was therefore used as a negative control. Interestingly, we observed that next to Efb-F also Efb-C and Ecb could not inhibit C3b binding to neutrophils (Figure e) or CHO-CR1 cells (Figure f).

In conclusion, incubation of neutrophils, CHO-CR1 cells and Jurkat-CRIg cells with Efb results in hampered C3b binding while Efb-C, Efb-F and Ecb are incapable to prevent this binding, which indicated that both the fibrinogen-binding N-terminus as well as the complement binding C-terminus are required.

C3b binding molecules prevent the interaction between C3b and cells

Above data suggest that Efb can bind to neutrophils or other cells (expressing complement receptors). However, attempts to demonstrate direct binding of Efb, Efb-C or Ecb to human cells were unsuccessful (data not shown). Nevertheless, the fact that pre-incubation of cells with Efb leads to inhibited binding of C3b, suggests that Efb binds to human cells most likely via its fibrinogen binding part. Since Ecb and Efb-C lack this fibrinogen binding part we tested whether pre-incubation of C3b with Efb-C and Ecb could result in hampered binding of C3b to neutrophils and CHO-CR1 cells. So, in contrast to the data above, C3b was pre-incubated with the C3d binding proteins before it was presented to neutrophils. Indeed we were able to show that pre-incubation of C3b with Efb-C and Ecb results in inhibition of C3b binding to these cells similar as shown for Efb (Figure 2a-b). Efb-F remains unable to inhibit this binding. Next,

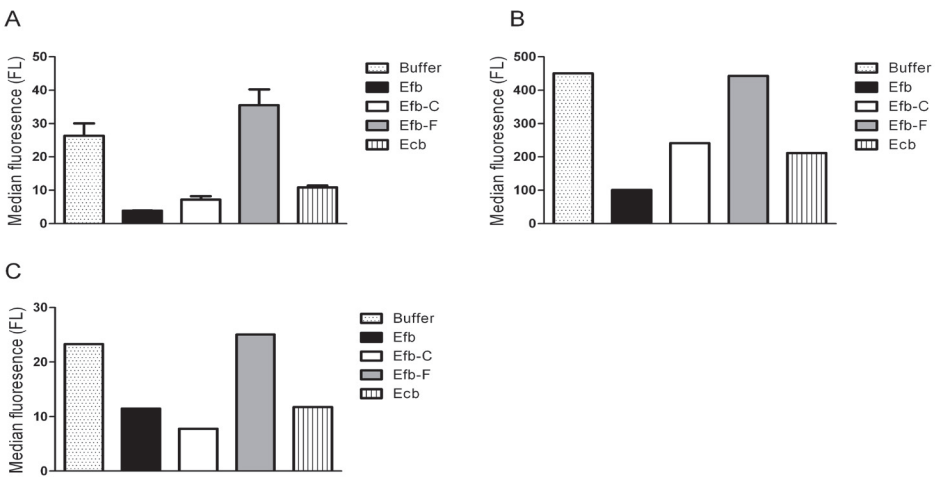

Figure 2: C3b binding molecules prevent C3b binding to cells

(a) Binding of C3b (15 µg/ml) pre-incubated with Efb, Efb-C, Efb-F or Ecb (all at 10 µg/ml) to freshly isolated neutrophils. (b) Binding of C3b (15 µg/ml) pre-incubated with Efb, Efb-C, Efb-F or Ecb (all at 10 µg/ml) to CHO-CR1 cells. (c) Disturbance of pre-bound C3b to neutrophils by Efb, Efb-C, Efb-F and Ecb (all at 10 µg/ml). Figure a represents the median ± SEM of two separate experiments while figure b and c are representative graphs of 2 separate experiments.

we wondered whether these molecules could also disturb binding of C3b that was already bound to neutrophils. Therefore, neutrophils were first incubated with C3b, washed and subsequently treated with Efb, Efb-C, Efb-F and Ecb. Figure 2c shows that all proteins accept Efb-F are able to disrupt the binding of C3b to neutrophils.

Thus, pre-incubation of C3b with Efb, Efb-C and Ecb inhibits C3b binding to cells. Next, all C3b binding proteins are able to disrupt the binding of C3b pre-bound to neutrophils.

Efb is unable to prevent iC3b binding to neutrophils or Jurkat-CRIg cells

Efb can both bind C3b and iC3b[10,11]. Since Efb effectively hampers C3b binding to neutrophils and Jurkat-CRIg cells we investigated whether Efb can inhibit iC3b binding to these cells as well. Neutrophils were incubated with Efb whereupon iC3b was added and binding was analyzed. Figure 3a shows that Efb is unable to inhibit iC3b binding to neutrophils. Similar results were obtained when iC3b binding to Jurkat-CRIg cells was determined in the presence or absence of Efb (Figure 3b).

So, although Efb binds to iC3b the protein is unable to prevent iC3b binding to neutrophils or Jurkat-CRIg cells.

Efb inhibits phagocytosis of C3b-coated particles

We showed that Efb and Ecb effectively prevent binding of soluble C3b to neutrophils and CHO-CR1 cells. To investigate the immune evasion properties in a more natural way, we determined whether Efb and Ecb could also prevent binding of C3b opsonized particles to cells rather than soluble C3b. Therefore, SRBC were opsonized with C3b, using C5 deficient human serum, and rosette formation with neutrophils and CHO-CR1 cells in the presence or absence of Efb or Ecb was analyzed. During these experiments, neutrophils and SRBC were both pre-incubated with Efb and Ecb. Figure 4a and b show that both Efb and Ecb are capable to prevent binding of opsonized SRBC to neutrophils or CHO-CR1 cells.

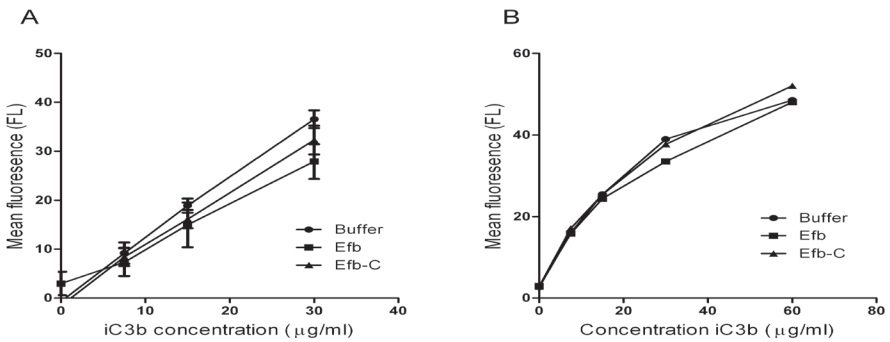

Figure 3: Efb does not inhibit iC3b binding to cells

(a) iC3b binding to freshly isolated neutrophils in the presence or absence of Efb or Efb-C (both at 10 μg/ml). (b) iC3b binding to Jurkat-CRIg cells in the presence or absence of Efb or Efb-C (both at 10 μg/ml). Figure a represents the mean ± SEM of three separate experiments while figure b is a representative graph of two separate experiments.

Recently, we showed that both Efb and Ecb are unable to inhibit phagocytosis of bacteria in the presence of human serum[11]. Since it seems that the fibrinogen binding part of Efb plays an important role in the prevention of C3b binding to neutrophils, we studied whether Efb is capable to prevent phagocytosis in the presence of fibrinogen. Bacteria were opsonized with human plasma in the presence or absence of Staphylococcal Complement INhibitor (SCIN) (positive control[11]), Efb, Efb-C, Efb-F and Ecb (all at 10 µg/ml) before freshly isolated neutrophils were added. Figure 4c shows that Efb, but not Efb-C, Efb-F and Ecb, potently inhibits phagocytosis similar to SCIN. To investigate whether this inhibition is strictly due to the presence of fibrinogen, bacteria were opsonized in 5% human serum supplemented with different concentrations of fibrinogen. Figure 4d shows that increasing amounts of fibrinogen result in higher inhibition by Efb.

In conclusion, Efb and Ecb both inhibit rosette formation. In addition Efb effectively inhibits phagocytosis in the presence of fibrinogen as well.

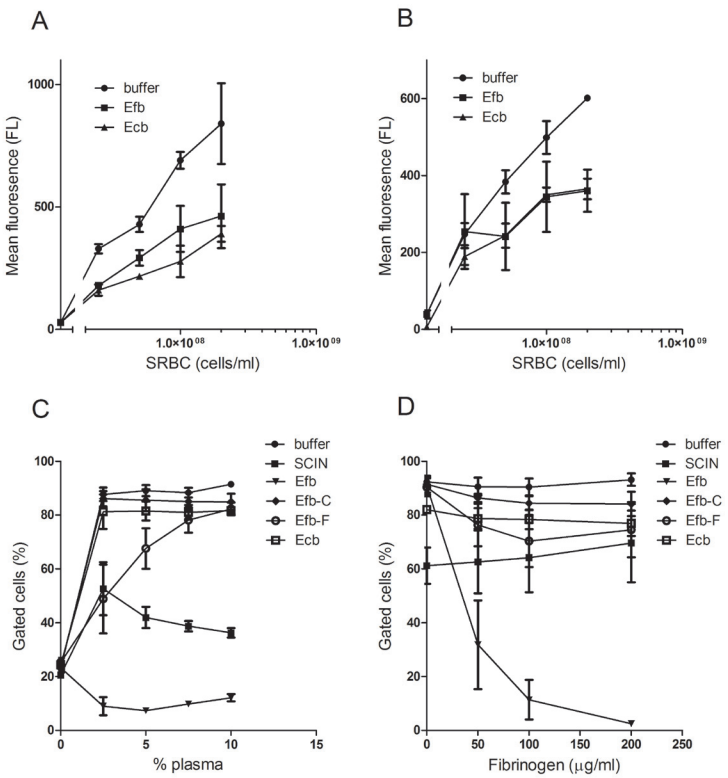

Figure 4: C3b binding molecules prevent rosette formation and phagocytosis
(a) Rosette formation with freshly isolated neutrophils in the presence or absence of Efb or Ecb (both at 10 µg/ml). (b) Rosette formation with CHO-CR1 cells in the presence or absence of Efb or Ecb (both at 10 µg/ml). (c) Phagocytosis of *S. aureus* strain KV 27 in plasma in the presence or absence of SCIN, Efb, Efb-C, Efb-F and Ecb (all at 10 µg/ml). (d) Phagocytosis of *S. aureus* strain KV 27 in 5% serum supplemented with fibrinogen in the presence or absence of SCIN, Efb, Efb-C, Efb-F and Ecb (all at 10 µg/ml). All figures represent the mean ± SEM of three separate experiments.

DISCUSSION

The human pathogen *S. aureus* secretes several small immune evasion molecules that enable the bacterium to survive of in the human host. Among these proteins several of them specifically inhibit the complement system[4,18] and several of them were shown to be able to evade recognition by complement receptors[13,19](Chapter 5). First, it was shown that Efb and Ecb, which both bind to the C3d domain of C3, C3b, iC3b and C3dg, effectively inhibit C3d binding to CR2, thereby preventing B-cell proliferation which presumably results in inhibition of the adaptive immune response[13](Chapter 5). Staphylococcal immunoglobulin-binding protein (Sbi) is another protein known to inhibit C3d binding to CR2[20,21]. Secondly, we recently showed that Staphylococcal Complement INhibitor (SCIN) prevents C3b recognition by CR1, CRIg and indirectly CR3[19]. SCIN effectively inhibits C3 convertases of all complement pathways[22]. Inhibition of the AP is achieved by formation of dimeric convertases[14] and this dimerization of convertases was shown to shield the binding place for CR1 and CRIg[19]. Here, we showed that Efb and Ecb hamper the binding of C3b to neutrophils, CHO-CR1 cells and to Jurkat-CRIg cells. Moreover, Efb and Ecb reduced rosette formation.

We showed that pre-incubation of human cells with Efb prevented binding of C3b, while pre-incubation of cells with Ecb or Efb-C did not result in inhibition. These data indicate that Efb is able to bind to cells while Efb-C and Ecb lack this ability. Efb binds to fibrinogen while Efb-C and Ecb cannot[11], since fibrinogen is present on human neutrophils[23] it is likely that Efb binds to this fibrinogen and stays attached to the cell. Unfortunately, we are still unable to detect binding of Efb to cells. This is probably due to technical limitations and further research is needed to investigate whether Efb indeed binds to cells. Efb and Ecb were known to specifically inhibit C3b-containing convertases, both proteins could inhibit C3b deposition via the AP up to 50% on the bacterial surface but they were unable to prevent C3b deposition via the CP/LP. Most importantly, both proteins seemed unable to prevent phagocytosis[11]. In contrast, here we do find that Efb effectively inhibits phagocytosis. This can be explained by the addition of fibrinogen which was absent in previous phagocytosis experiments. Since we showed that pre-incubation of neutrophils with Efb resulted in decreased C3b binding, it would be interesting to see whether pre-incubation of neutrophils with Efb results in inhibition of phagocytosis as well. C3b is known to directly bind the target surface via its thioester domain[24] therefore, inhibition binding and phagocytosis of C3b opsonized particles by Efb and Ecb will be highly relevant in *S. aureus* pathogenesis.

As already described above Efb is unable to prevent iC3b binding to cells while it effectively reduced binding of C3b to cells even though it is known that Efb binds to C3b and iC3b with similar affinity[10,11]. Efb binds to the C3d domain of C3b, and this binding is proposed to induce conformational changes in the C3b molecule[10]. Since we showed that C3b binding to CR1 and CRIg is reduced in the presence of Efb we hypothesize that these conformational changes result in hampered recognition by CR1 and CRIg. Since the co-structure of Efb together with C3b is not available yet we are unable to support this hypothesis further. The orientation of C3dg in the iC3b molecule is completely different compared to the C3b molecule[24] therefore, it is possible that binding of Efb to the C3dg domain of iC3b is unable to introduce conformational changes or binding of Efb to iC3b thus not result in conformational changes that disrupt the recognition by CR3 and CRIg. Unfortunately, the binding sites of CRIg to the iC3b molecule are unknown so currently there is no extra information about amino acids that might undergo conformational changes in the C3b molecule after Efb or Ecb are bound to it.

Together these data indicate that expression of Efb and Ecb by *S. aureus* is a very effective way to evade the human immune system. The proteins not only effectively inhibit C5a generation and thereby prevent neutrophils migration towards the side of infection[11], they also inhibit activation of the adaptive immune system preventing C3d binding to CR2[13](Chapter 5). Here we show that next to this, Efb prevents phagocytosis as well. Since the mechanism of action for Efb is almost completely unravelled it might be worthwhile to study the abilities to use this protein in novel anti-inflammatory therapies as already occurs for another secreted protein of *S. aureus* staphylokinase[25,26].

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Chapter 7

Secreted immune evasion molecules contribute to *S. aureus* pathogenesis *in vivo*

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Manuscript in preparation

ABSTRACT

The human pathogen *Staphylococcus aureus* produces a number of small, secreted proteins that specifically block key elements of the host innate immune response. At present, we know that *S. aureus* secretes over 15 different innate immune inhibitors that all seem to target a different step in the inflammatory response. Due to the human-specificity of most such proteins, the role of these factors in bacterial pathogenicity has not been confirmed *in vivo*. We recently described two non-human specific immune inhibitors in *S. aureus:* Extracellular complement binding protein (Ecb) and Extracellular fibrinogen binding protein (Efb). Ecb and Efb are complement inhibitors that block neutrophil migration towards the site of infection by preventing formation of the complement-derived chemoattractant C5a. Here, we used a targeted mutagenesis approach to study the role of Ecb and Efb in *S. aureus* pathogenesis. In contrast to wild-type *S. aureus* strain Newman, an isogenic mutant lacking Ecb and Efb (ΔEcbΔEfb) was unable to prevent C5a generation upon incubation of bacteria with human serum. Also, mutant bacteria showed impaired survival in human whole blood. In a mouse pneumonia model, Ecb and Efb suppressed neutrophil influx into the lungs and promoted bacterial survival. In a systemic infection model, we showed that Ecb and Efb contribute to *S. aureus* persistence and abscess formation in kidneys. Ultimately, mice infected with wild-type bacteria experienced significant higher mortality than those infected with the isogenic ΔEcbΔEfb mutant. In conclusion, we demonstrate that these secreted immune evasion proteins produced by *S. aureus* can be validated as important virulence factors *in vivo*.

INTRODUCTION

Staphylococcal aureus is a commensal of human skin and nares but also an important human pathogen causing severe acute and chronic infections. Infections with *S. aureus* can be community- and hospital derived, often they are initiated by bacterial invasion of tissue or bloodstream via trauma, surgical wounds, or medical devices[1]. In the last decade it has become clear that *S. aureus* secretes a large arsenal of immune evasion molecules. All these molecules have unique properties and block the human immune system at different steps[2-4]. Some proteins inhibit the bactericidal effect of defensins[5,6], while others interfere with neutrophil rolling on the epithelial lining[7] or neutrophil migration towards the site of infection. Chemotaxis inhibitory protein of *S. aureus* (CHIPS) binds to the C5a Receptor (C5aR) and the formylated peptide receptor thereby effectively inhibiting neutrophil migration[8,9]. *S. aureus* is an effective inhibitor of the complement system as well. Staphylococcal complement inhibitor (SCIN) inhibits the C3 convertase enzymes thereby preventing phagocytosis and C5a generation[10]. Because CHIPS, SCIN and most other immune evasion molecules are highly human specific, their relative contribution to the pathogenesis and virulence of *S. aureus* infections is currently unknown.

Lately, we described the complement inhibitory properties of Extracellular fibrinogen binding protein (Efb) and its homologue Extracellular complement binding protein (Ecb). The N-terminus of Efb was known to bind fibrinogen and thereby modulate platelet functioning[11,12]. Recently, it was shown that Ecb and the C-terminal domain of Efb specifically bind and inhibit C3b-containing convertases. This way, Ecb and Efb effectively block the C5 convertases that mediate C5a generation and neutrophil migration[13]. Interestingly, both proteins turned out not to be human specific giving us the opportunity to examine their function *in vivo*. The anti-inflammatory properties of purified Ecb were demonstrated in a mouse model for immune complex disease: pre-treatment of mice with Ecb completely blocked the C5a dependent migration of neutrophils[14]. Here, we use a targeted mutagenesis approach to study the role of Ecb and Efb in *S. aureus* pathogenesis. We demonstrate that Ecb and Efb are important virulence factors that contribute to bacterial pathogenesis and lethality in mice.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions

S. aureus and *E. coli* strains and plasmids are listed in supplementary table 1. *E. coli* was grown on Luria-Bertani (LB) medium or agar at 37ºC. *S. aureus* was cultured on LB, Todd-Hewit or LK (containing 10 g/L tryptone, 5g/ml yeast extract, 7 g/ml KCL) medium/agar at 37ºC. Carbenicillin (50 µg/ml), tetracycline (5µg/ml), kanamycin (50 µg/ml), neomycin (50 µg/ml), erythromycin (5 µg/ml), lincomycin (25 µg/ml) and chloramphenicol (10 µg/ml) (all from Sigma-Aldrich) were used for plasmid selection.

Generation of Ecb and Efb mutants and complementation

The *ecb* (330bp) and *efb* (498 bp) genes were disrupted by allelic replacement as described previously[15]. In short, the first 216 bp of *ecb* plus the 515 bp upstream region were amplified using EcbUPfor/EcbUprev and the last 109 bp of *ecb* plus 1444 bp downstream *ecb* using EcbDOWNfor/EcbDOWNrev (oligonucleotides are listed in supplementary table 2, restriction sites are underlined). The tetracycline resistance gene from plasmid pDG1513 was amplified using the primers TetF/TetR. The three fragments were digested with BamH/NotI, KpnI/EcoRI and NotI/KpnI respectively and ligated into EcoRI/BamHI-digested pAZ106[16] forming pAZ106::Ecb-Tet. Allelic replacement of *efb* was achieved similarly by amplification of the the first 271 bp of *efb* plus its 670 bp upstream region using primers EfbUPfor/EfbUPrev and the last 241 bp of *efb* plus 443 bp downstream EfbDOWNfor/EfbDOWNrev. The tetracycline was used for single gene insertions while the kanamycine gene from plasmid pDG792 was amplified using KanF/KanR for the construction of the double mutant (NewmanΔEcbΔEfb). The three fragments were digested as described above and ligated forming pAZ106::Efb. The pAZ106::Ecb-Tet and pAZ106::Efb-Tet plasmids were transformed to *E. coli* Top10F and analyzed by restriction digestion analysis. Correct plasmids were first transformed into *S. aureus* RN4220 and subsequently transduced into *S. aureus* strain Newman with Φ11 as described previously[17]. Plasmid pAZ106::Efb-kan was transduced into *S. aureus* Newman or the ΔEcb mutant to generate the ΔEcbΔEfb double knock-out. Allelic replacements were confirmed by PCR, sequencing and immunoblotting.

For complementation analysis, the *ecb* or *efb* gene including the 300 bp upstream (containing the start codon) and 50 bp downstream (containing the stop codon) regions was amplified using primers EcbF/EcbR or EfbF/EfbR. The fragments were cloned as a HindIII/EcoRI fragment into pCU1[18], and transformed to *E. coli* strain Top10F'. Plasmids were validated by sequencing and correct plasmids were transduced into *S. aureus* NewmanΔEcb, NewmanΔEfb or NewmanΔEcbΔEfb and selected on TH agar plates containing chloramphenicol. Complementations were confirmed by colony PCR and immunoblotting.

Silverstaining and Immunoblot

Wild-type and mutant *S. aureus* strains were first cultured on LB-agar plates with appropriate antibiotics, then overnight cultures were prepared in Iscove's Modified Dubbecco's Medium (IMDM) (Lonza, Biowhithaker) without antibiotics. Subsequently, bacteria were diluted to an OD_{660} of 0.05 and grown until OD_{660} of 0.5. Supernatants were collected by centrifugation and concentrated 33 times by incubating supernatants with 10% Trichloroacetic acid (TCA) (Sigma) overnight at 4ºC. Samples were run on SDS-PAGE gel and expression was either detected by silverstaining or by immunoblotting. For immunoblotting, Ecb was detected by rabbit anti-Ecb antibodies (1 µg/ml) (produced by Biogenes, Berlin, Germany) followed by PO-conjugated goat anti-rabbit (Southern biotech). Efb was detected using sheep-anti Efb antibodies (1 µg/ml) (kindly provided by Jan-Ingmar Flock) followed by peroxidase (PO) conjugated donkey anti-sheep antibodies (Sigma).

Whole blood phagocytosis and survival

Whole blood phagocytosis was performed as described previously[19]. In short: Freshly isolated human blood was anticoagulated using lepirudin (Refludan, Schering, Kenitworth, NJ) and pre-incubated at room temperature with the purified proteins Staphylococcal Superantigen-like (SSL) 10, Ecb or Efb (protein pre-

paration as described previously[20,21]). Blood was incubated with FITC-labeled *S. aureus* strain KV27 (3 x 10^7) for 25 minutes at 37°C. Blood was subsequently lysed by FACS Lysing solution (BD biosciences) and phagocytosis was analyzed by flow cytometry. To analyze bacterial survival in whole blood, overnight cultures of wild-type and mutant *S. aureus* were diluted to an OD_{660} of 0.05 in fresh THB and grown with shaking at 37°C to an OD_{660} of 0.8. Bacteria were diluted to a concentration of 5 x 10^6 CFU/ml and 50 µl bacteria were incubated with 50 µl freshly isolated blood and 100 µl RPMI 1640 (Life Technology, Carlsbad) containing 0.05% HSA (Sanquin) for 0-6 hours (shaking, 37°C). Blood was lysed with 1 ml of ice-cold H_2O and bacterial survival was enumerated by plating serial dilutions on TH agar plates.

C5a generation

S. aureus strains wild-type Newman and NewmanΔEcbΔEfb were grown as described above. Fifty µl bacteria (5 x 10^6 CFU/ml) were incubated with 150 µl 30% human pooled serum for 0-8 hours at 37°C shaking, whereupon the supernatants were collected by centrifugation. Subsequently, collected supernatants were tested for the presence of C5a. U937-C5a receptor cells were incubated with the supernatants and calcium mobilization was measured as described[22]. The inoculum was always verified by plating and colony enumeration.

Murine pneumonia model

Overnight cultures of *S. aureus* Newman and *S. aureus* NewmanΔEcbΔEfb were diluted 1:100 in fresh THB and grown with shaking at 37°C to an OD_{660} of 0.8. Bacterial cultures were centrifuged and resuspended in PBS to the desired concentrations. The inoculum was verified by plating and colony enumeration. The pneumonia model was performed as described previously[23] with minor modifications. Following anesthesia with ketamine and xylazine, 8-weeks old female CD1 mice (Charles River Laboratories) were inoculated with 10 µl of 2x 10^8 CFU *S. aureus* in each nare. Animals were held upright for 1 minute and recovery from anesthesia was monitored. Animals were euthanized by CO_2 inhalation at 6 and 24 hours post infection. Blood was collected by cardiac puncture and lungs were lavaged with PBS to collect the broncho-alveolar lavage fluids. Right lungs and noses were excised and bacterial loads were enumerated by plating homogenized tissues in serial dilutions on THA. To analyze neutrophil influx, mice were euthanized 6 hours post infection and lungs were inflated with 10% formalin. The trachea was closed and formalin-inflated lungs were excised and parafin-embedded. Deparrafinized sections were stained for myeloperoxidase using rabbit anti-human MPO (Dako) and Alexa 488 goat anti rabbit antibodies (Molecular Probes).

Murine intravenous infection model

Bacteria were prepared as described above. Eight-weeks old female Balb/c mice (Charles River Laboratories) were infected with 2 x 10^7 CFU *S. aureus* by intravenous inoculation via the lateral tail vein. To assess mortality, mice were checked daily for clinical signs and body weight measurements were taken. In separate experiments, mice were sacrificed 2 or 10 days post infection by isofluorane inhalation. Blood was collected from the portal vein; lungs, heart, kidneys, spleen and liver were excised. All organs and the right kidney was homogenized and bacterial loads were enumerated by plating serial dilutions on THA. To asses abscess formation, the left kidney was fixed in 10% formalin and embedded in paraffin. Paraffin-embedded kidneys were sectioned 3 and 6 µm from the organ center. Sections were stained with hematoxilin and eosin and examined by microscopy.

Statistics

Differences in bacterial counts, C5a generation, and whole blood survival were evaluated by Student's T test. Significance was determined as $P < 0.05$. Survival curves were evaluated by the Wilcoxon's rank-sum test.

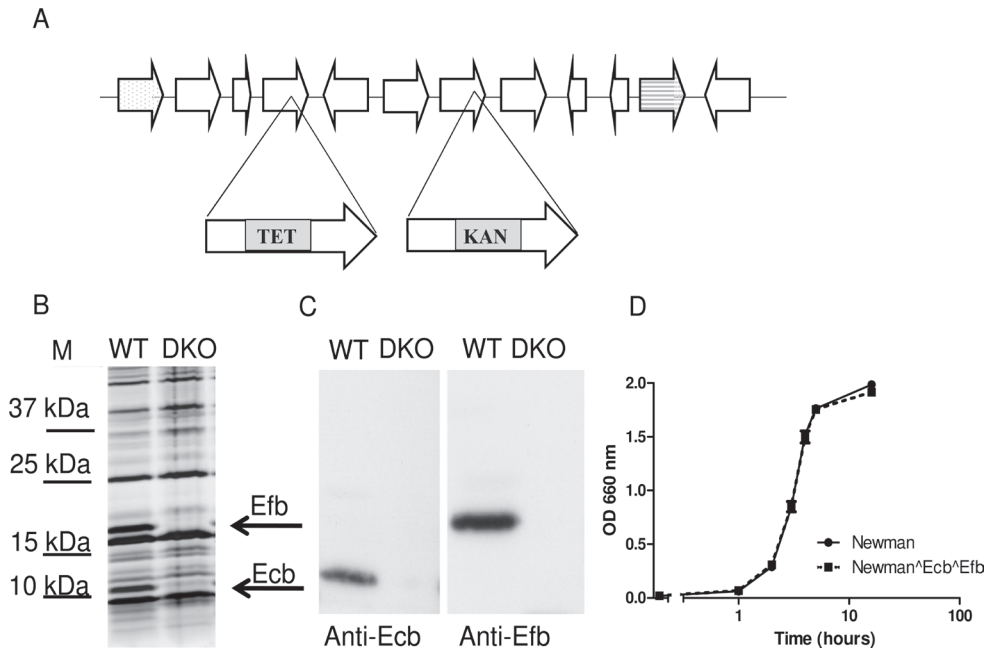

Figure 1: Construction of *S. aureus* strain NewmanΔEcbΔEfb
(a) Schematic representation of the *S. aureus* NewmanΔEcbΔEfb strain. A tetracycline cassette was incorporated in the *ecb* gene while a kanamycine cassette was incorporated in the *efb* gene. (b) Analysis of Ecb and Efb expression detected by SDS-PAGE. *S. aureus* strain Newman and NewmanΔEcbΔEfb were grown in IMDM until OD=0.5, supernatants were collected and concentrated via TCA precipitation. (c) Analysis of Ecb and Efb expression in concentrated supernatants by immunoblotting using anti-Ecb and anti-Efb antibodies. *S. aureus* strain NewmanΔEcbΔEfb neither produced Ecb nor Efb. (d) Growth curves of *S. aureus* strain Newman and NewmanΔEcbΔEfb. Both strains were grown over night in TH broth, subsequently they were diluted to an OD_{660} of 0.05 and OD was measured every hour. Both strains grew equally. Figure 1b-c are representatives of 3 separate experiments while figure 1d represents the mean ± SEM of three experiments.

RESULTS

Mutagenesis and complementation of Ecb and Efb in *S. aureus* Newman

To explore the functional role of the immune evasion molecules Ecb and Efb in *S. aureus* pathogenesis, isogenic mutants of *S. aureus* strain Newman were generated by allelic replacement. Both proteins effectively inhibit C5a generation *in vitro*; therefore we constructed a double mutant strain (ΔEcbΔEfb) (Figure 1a) in addition to the single mutant *S. aureus* Newman variants (ΔEcb and ΔEfb) (data not shown). The isogenic mutants were complemented by introduction of the Ecb gene or the Efb gene on an expression plasmid[24]. The insertion of the tetracycline- or kanamycin cassette for Ecb or Efb respectively was confirmed by PCR (data not shown) and sequencing (data not shown). To confirm the absence of Ecb and Efb protein expression in NewmanΔEcbΔEfb we either analyzed the expression of both proteins by SDS-PAGE followed by silverstaining (Figure 1b) or by immunoblotting (Figure 1c), both figures show the absence of expression of both proteins. All other single mutants and complementary strains were analyzed by immunoblot as well (data not shown). In addition, we analyzed the growth ability of the wild type *S. aureus* Newman strain and the *S. aureus* mutant strain. Figure 1d shows that mutation of Ecb and Efb does not result in impaired growth. Similar results were obtained for the single mutants and the complementary Newman strains (data not shown).

In conclusion, we successfully constructed a *S. aureus* Newman mutant strain (ΔEcbΔEfb).

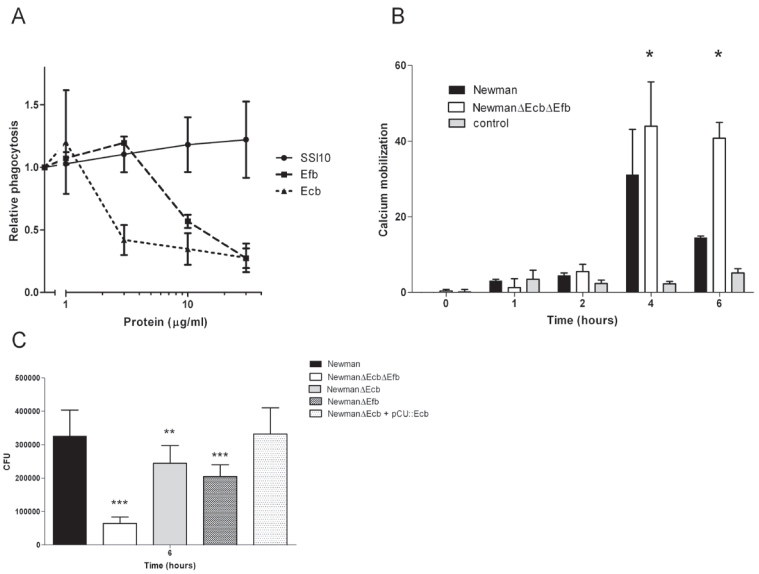

Figure 2: Ecb and Efb are essential for whole blood survival of *S. aureus* strain Newman
(a) Whole blood phagocytosis in the presence or absence of SSL10, Ecb or Efb. Ecb and Efb inhibit phagocytosis of *S. aureus* strain KV27. (b) C5a generation by *S. aureus* strain Newman and NewmanΔEcbΔEfb. Fifty µl bacteria (5 x 10^6/ml) was incubated with 30% human serum and C5a generation was measured via calcium mobilization of U937 C5aR cells. NewmanΔEcbΔEfb incubated with human serum generated more C5a than wild type Newman. *P<0.05 according to the student-T test. (c) Fifty µl bacteria (5 x 10^6/ml) (wild type Newman, NewmanΔEcbΔEfb; NewmanΔEcb; NewmanΔEfb and NewmanΔEcb plus pCU1::Ecb) was incubated with 25% human blood and bacterial survival was detected after six hours. **P<0.01, ***P<0.005 according to the student T-test. All figures represent the mean ± SEM of three separate experiments.

Ecb and Efb are essential for staphylococcal C5a inhibition and bacterial survival in whole blood

Recombinant Ecb and Efb can inhibit C5a generation in serum[25], to verify whether both proteins can inhibit C5a generation in whole blood as well, we investigated their inhibitory properties in a C5a dependent whole blood phagocytosis[26]. Figure 2a shows that phagocytosis is inhibited in the presence of both Ecb or Efb while the presence of the negative control SSL10[27] did not affect phagocytosis. To determine whether expression of Ecb and Efb by *S. aureus* is important for staphylococcal C5a inhibition we compared the C5a generation by wild type *S. aureus* strain Newman with the ΔEcbΔEfb mutant. We observed that significantly less C5a is generated by the wild type bacteria compared to the ΔEcbΔEfb mutant (Figure 2b). In addition, no spontaneous generation of C5a in serum incubated at 37°C up to 6 hours was observed (Figure 2b, control).

Above data indicate that expression of Ecb and Efb are important to prevent phagocytosis and C5a generation in whole blood. To investigate whether expression of Ecb and Efb enhance survival of *S. aureus* in whole blood we incubated wild-type Newman and several isogenic mutants with whole blood for 6 hours and analyzed bacterial survival by plating and CFU enumeration. Figure 2c shows that survival of NewmanΔEcbΔEfb is significantly reduced compared to wild type Newman. Also the single mutants NewmanΔEcb and NewmanΔEfb show a significant impaired survival. The survival ability of NewmanΔEcb could be restored by complementation.

Thus, recombinant Ecb and Efb inhibit phagocytosis. In addition, expression of Ecb and Efb by *S. aureus* strain Newman inhibits C5a generation and is essential for whole blood survival.

Ecb and Efb block neutrophil influx resulting in staphylococcal pneumonia

Complement activation and subsequent neutrophil recruitment are important steps in pulmonary clearance of bacteria[28]. To investigate whether *S. aureus* can delay neutrophil responses by secretion of complement inhibitors, mice were inoculated via the intranasal route with either 4 x 10^8 CFU of *S. aureus* Newman or its isogenic ΔEcbΔEfb mutant. At these doses, animals did not succumb to infection. Animals were sacrificed 6 and 24 hours post infection, and bacterial loads in lavage and lungs were determined (Figure 3a). Six hours post infection we found similar amounts of bacteria in the lungs and lavage. However, after 24 hours significantly more bacteria were recovered from lungs of mice infected with wild-type than knock-out bacteria, indicating that Ecb and Efb prevent bacterial clearance from the lungs. To determine whether Ecb and Efb prevent neutrophil responses, experiments were repeated and formalin-fixed lung tissues were collected 6 hours after inoculation. Lungs were examined by immunostaining using the neutrophil-specific marker myeloperoxidase. Figure 3b shows a marked increase of neutrophils in lungs infected with knock-out but not wild-type bacteria.

Our data demonstrate that Ecb and Efb prevent neutrophil influx *in vivo* and thereby promote *S. aureus* pneumonia.

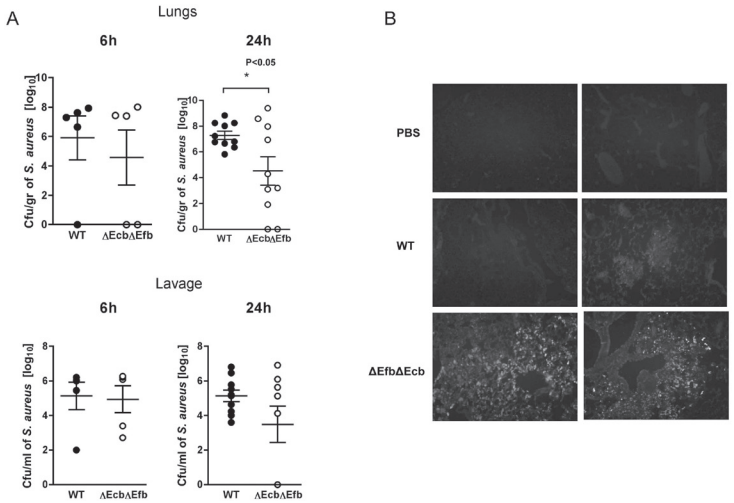

Figure 3: Deletion of Ecb and Efb increases neutrophil influx in a mouse model of staphylococcal pneumonia

(a) Intranasal infection with 4x10^8 CFU *S. aureus* WT results in higher bacterial loads in the lungs than with the ΔEcbΔEfb mutant at 24 h but not at 6h. No significant differences were found in the lavage. Five and ten mice per group were used at 6 h and 24h respectively. (b) Increased neutrophil influx in lung tissues of mice infected with the ΔEcbΔEfb mutant compared to *S. aureus* WT at 6h. Neutrophils were detected in formalin-fixed lung tissues using the neutrophil-specific marker myeloperoxidase. Top: PBS-infected mice. Five mice were used per group.

Ecb and Efb are required for staphylococcal virulence and abscess formation

To study the role of Ecb and Efb in invasive staphylococcal infections, eight-weeks old female BALB/c mice were infected with 1.5 x 10^7 CFU of *S. aureus* Newman or its isogenic ΔEcbΔEfb mutant via intravenous inoculation. Clinical signs and body weight were monitored daily. We observed that Ecb and Efb play a role in staphylococcal virulence, since mortality rates due to infection were much higher in mice infected with wild-type bacteria than ΔEcbΔEfb mutants (Figure 4a). Also, mice infected with wild-type bacteria showed more weight loss (Figure 4b). In a different experiment, we sacrificed mice 2 or 10 days post infection. Organs were removed, homogenized and bacterial load was enumerated by plating. After 10 days of infection, we observed higher bacterial loads in kidneys infected with wild-type bacteria than ΔEcbΔEfb mutants (*P* =0.028) (Figure 4c). Also in the heart, higher amounts of wild-type than knock-out bacteria were observed, although this was not statistically significant (*P*=0.052). No differences in bacterial loads were observed after 2 days of infection. Bacteria were also found in lung, liver and spleen tissues but no differences between wild-type and knock-out bacteria was observed (data not shown). As shown previously, no bacteria were found in the blood[29].

Previous studies used a similar model to study staphylococcal abscess formation in kidneys. Visible examination of excised kidneys revealed much more abscesses in kidneys of mice infected with wild-type *S. aureus* (Figure 4d). Examination of HE-stained kidney section also revealed that mice infected with wild-type *S. aureus* contained more abscesses in their kidneys than mice infected with ΔEfbΔEcb *S. aureus* (*P*=0.04) (Figure 4e).

Altogether these data demonstrate that Ecb and Efb are staphylococcal virulence factors that contribute to bacterial persistence and abscess formation in kidneys.

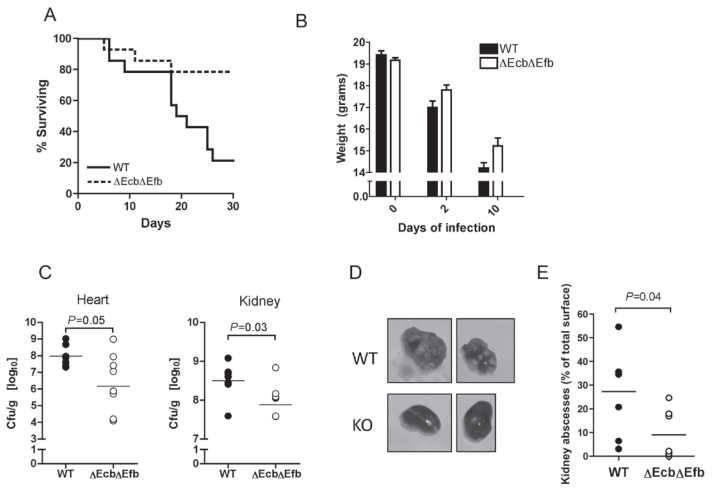

Figure 4: Ecb and Efb are required for staphylococcal virulence and abscess formation

(a) Mice were infected with 1.5 x 10^7 CFU of *S. aureus* WT or the ΔEcbΔEfb mutant via intravenous inoculation. *a.* Higher mortality of mice infected with *S. aureus* WT than with the ΔEcbΔEfb mutant (*P*=0.007, n=14) *b.* Increased weight loss in mice infected with *S. aureus* WT than with the ΔEcbΔEfb mutant. (c) Bacterial loads in heart and kidney of mice infected with wild type *S. aureus* or it isogenic mutant ΔEcbΔEfb at day 10 (WT: n=7, KO:n=8). (d) Representative pictures of kidneys of mice infected with *S. aureus* WT or the ΔEcbΔEfb mutant 10 days post infection. (e) Quantitative determination of kidney abscesses from of H&E stained kidneys at day 10 (WT: n=7, KO:n=8).

DISCUSSION

S. aureus is known to produce several virulence factors such as adhesins and toxins[30]. The importance of adhesins was already shown *in vivo*[31,32] and in addition, also the function of toxins is generally accepted[33]. Lately, it was shown that *S. aureus* produces a large arsenal of secreted immune evasion molecules as well[34-36]. The importance of these molecules *in vivo* is difficult to study. First, most of these proteins turned out the be highly human specific which limits animal studies[37,38]. Second the timing of secretion of these molecules is more difficult than for surface-bound proteins where washing of bacteria before inoculation does not influence expression of the proteins on the surface. The amount of immune evasion molecules secreted by *S. aureus* might look redundant, but here we were able to show that deletion of only two molecules decreases pathogenesis significantly. Secretion of Ecb and Efb by *S. aureus* strain Newman turned out to be important for bacterial persistence in lungs. A large variability in the amount of recovered mutant bacteria was observed which can either be explained by a steep dose-response curve (data not shown) or we might look at a local infection since we only recovered the bacteria from one lung. The clearance of bacteria in the lungs is in consensus with a marked increase of neutrophils in lungs infected with mutant bacteria compared to lungs infected with wild type bacteria. C5a generation in the lung is of high importance for the clearance of *S. aureus* and *Pseudomonas aeuginosa*[39]. Due to technical limitations we were unable to detect C5a generation in the lung, but since Ecb and Efb both inhibit C5a generation very effectively[40] it is likely that the increase of neutrophils in lungs infected with mutant bacteria is a result of the presence of higher amounts of C5a.

Secretion of Ecb and Efb is shown to be of high importance for *S. aureus* pathogenesis in a renal abscess model. Mice infected with wild type bacteria showed a higher mortality, more weight loss, and persistence of bacteria in the kidney and the hearth compared to mice infected with the ΔEcbΔEfb mutant. Moreover, a higher amount of abscess formation was observed in the kidney of mice infected with wild type *S. aureus* compared to mice infected with the isogenic mutant. Lately, it was shown that abscess formation in this specific model involves neutrophil recruitment[41]. In addition, mice infected with a *S. aureus* mutant lacking expression of Extracellular adherence protein (Eap), which prevents neutrophil adhesion and extravasation[42], developed less kidney abscesses[43].

All these data show that expression of Ecb and Efb are crucial for *S. aureus* pathogenesis *in vivo* which is most likely C5a dependent but the importance of the fibrinogen binding part of Efb cannot by excluded. Efb binds fibrinogen[44] and is known to modulate platelet function[45]. Moreover, it was shown that an Efb mutant is less virulent in a wound infection model bases on a deep subcutaneous incision[46]. Here, we show the importance of secreted immune molecules in *S. aureus* pathogenesis in mice. Although mice are no natural reservoir of *S. aureus* we do think that immune evasion molecules are important during human infections as well since these molecules are present in almost all human isolates. The renal abscess model might be comparable with the formation of skin infection, were expression of Efb and Ecb probably block neutrophil migration resulting in bigger and more abscesses. In addition, pneumonia becomes an emerging disease caused by *S. aureus*[47], our data indicate that expression of Ecb and Efb will result in lack of neutrophil migration in the lungs which will be a disadvantage to the outcome of the patient.

The emergence of methicillin-resistant strains (MRSA) in the hospital and the emergence of the community-associated MRSA (CA-MRSA) make the bacterium a real public threat. CA-MRSA strain USA300 has become an epidemic strain in the USA[48]. To fight this epidemic the

need for vaccines is raising. Lately, it is shown that a vaccine against Eap turned out to be very successful in mice[49]. We show that mutation of two secreted immune evasion molecules reduces *S. aureus* pathogenesis significantly. Therefore, the best approach for vaccination would probably be a vaccine against surface proteins as well as small secreted immune evasion molecules.

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Chapter 8

Summarizing Discussion

The struggle of the fittest: pathogenic bacteria versus the human immune system

Staphylococcus aureus secretes a large number of immune modulating proteins to resist the human immune response. A vast majority of these evasion molecules are involved in complement inhibition. In this thesis I explored the complement inhibitory strategies of Staphylococcal complement Inhibitory (SCIN), Extracellular fibrinogen binding protein (Efb) and Extracellular complement binding protein (Ecb), three small secreted proteins of *S. aureus*. Although SCIN has similar structural features as Efb and Ecb (Figure 1), the complement-inhibitory mechanism of SCIN turned out to be completely different from Efb and Ecb. The fact that Efb and Ecb are not human specific (in contrast to SCIN) enabled us to proof the importance of these small complement inhibitory proteins in *S. aureus* pathogenesis *in vivo*.

Complement evasion by *S. aureus*

The human pathogen *S. aureus* can cause a variety of infections. Infections can be hospital- or community acquired and they range from superficial skin lesions such as boils and food-poisoning to more serious infections such as pneumonia, meningitis and endocarditis[1]. In the last decades it has become clear that, next to expression of adhesins and toxins, *S. aureus* secretes a large arsenal of immune modulating proteins. Among these immune evasion molecules, a large majority is targeted against the complement system[2-7]. One of these complement inhibitory proteins secreted by *S. aureus* is SCIN. SCIN binds to and stabilizes the C3 convertase of the classical pathway (CP), the lectin pathway (LP) and the alternative pathway (AP), thereby inhibiting the cleavage of C3 which results in effective inhibition of phagocytosis and formation of C5a[8]. Recently, the co-structure of the SCIN-convertase complex revealed that SCIN forms dimers of the AP convertase in fluid phase by bridging the C3b molecule and Bb molecule of the first convertase with the C3b molecule of the second convertase. The stabilization of the AP C3 convertase (C3bBb) by SCIN is essential for inactivation of the enzyme[9]. The downside of this stabilization seems that many C3b molecules (those within the C3 convertases) are still present on the bacterial surface and could trigger phagocytosis. To investigate the biological role of convertase dimerization by SCIN we made a SCIN mutant that is unable to form dimeric convertases since it lacks the binding site for the second C3b molecule (SCINΔC3b$_2$) (Chapter 2). We found that formation of dimeric convertases is not essential to inhibit complement activation on the bacterial surface since C3b deposition was inhibited equally well by SCIN and SCINΔC3b$_2$. On the other hand, we determined that formation of dimeric convertases by SCIN is essential to inhibit phagocytosis completely: C3b molecules incorporated in the dimeric convertase are no longer recognized by complement receptor (CR) 1 and Complement Receptor of the ImmunoGlobulin superfamily (CRIg)[10]. Moreover, we observed that stabilization of convertases by SCIN prevented C3b degradation by factor H and factor I, resulting in inhibition of C3b to iC3b conversion on the bacterial surface. This indirectly inhibits recognition of bacteria by the other important complement receptor on phagocytic cells, CR3 [10]. The fact that SCIN inhibits complement activation via convertase inhibition and in addition prevents recognition of the bacterium by CR1 and CRIg makes this molecule a very potent immune evasion protein.

Efb was known to bind to fibrinogen and C3b[11-14], therefore we hypothesized that Efb could play an important role in complement inhibition by *S. aureus*. In Chapter 3 we describe the complement inhibitory properties of Efb and also identified a C-terminal homologue of Efb, which we named Ecb. Both proteins bind to the C3d region of C3b thereby inhibiting C3b-containing convertases: the C3 convertase of the AP (C3bBb), the C5 convertase of the CP/LP (C3bC4b2a) and the C5 convertase of the AP (C3b$_2$Bb). Both proteins potently inhibit C5a generation and are, in contrast to SCIN, not human specific. The latter enabled us to test the activity of these molecules *in vivo*. We observed that Ecb completely inhibits C5a dependent

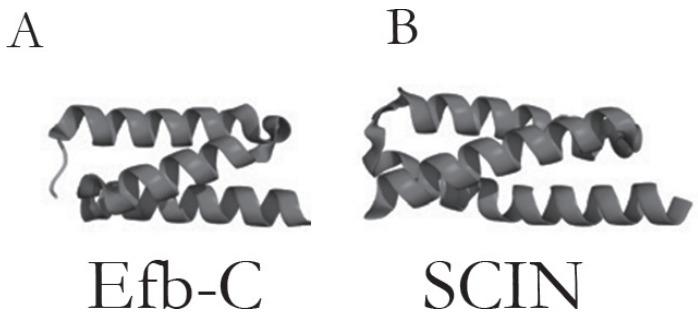

Figure 1: Structures of Efb-C and SCIN
Structures of Efb-C (a) and SCIN (b). Both Efb-C and SCIN have a three-helix bundle structure[49].

neutrophil migration in a mice model[15]. To investigate the molecular mechanism by which Ecb inhibits C3 and C5 convertases we used an Ecb mutant lacking the ability to bind the C3d domain of C3b. Binding of Ecb to C3b turned out to be essential to inhibit C5 convertases since the Ecb mutant was unable to prevent C5a generation. Furthermore, we observed that binding of Ecb to the C3b molecule resulted in enhanced binding of factor B (fB) to the C3b molecule which prevented formation of active convertases since the conversion of fB into Bb was blocked (Chapter 4). Following the discovery of the mechanisms of action for Efb and Ecb on C3b-containing convertases, we investigated the effect of Ecb on the adaptive immune system. C3 is a key player in the innate immune response but its degradation product C3d is an important stimulant for the adaptive immune system as well. Because the Ecb binding site to C3d is close to the predicted CR2 binding site, we explored whether Ecb is able to inhibit the adaptive immune response as well. Indeed we were able to show that Ecb inhibits the binding of C3d to CR2 (Chapter 5).

Although Efb has a fibrinogen binding part which is not present in the Ecb molecule their functions turned out to be similar, they both inhibit complement activation via C3b-containing convertases and C3d binding to CR2. Preliminary data showed that Efb might be involved in C3b binding to human cells. To identify possible extra functions of Efb due to its fibrinogen binding part, we studied the role of Efb and Ecb in C3b binding to cells. In Chapter 6 we showed that pre-incubation of neutrophils, CHO-CR1 cells and jurkat-CRIg cells with Efb prevented the binding of fluid phase C3b while Efb-C and Ecb lack this function. In contrast, pre-incubation of C3b with Efb, Ecb and Efb-C all resulted in hampered C3b binding to cells. This indicated that Efb is able to bind to cells while Ecb and Efb-C cannot. In addition, we showed that Efb and Ecb prevent binding of C3b-coated erythrocytes to neutrophils and CHO-CR1. In addition we were able to show that Efb is a potent inhibitor of phagocytosis in the presence of fibrinogen.

Finally, we were able to show that Ecb and Efb are staphylococcal virulence factors that contribute to *S. aureus* pathogenesis (Chapter 7). We use a targeted mutagenesis approach to study the role of Ecb and Efb in *S. aureus* pathogenesis. We generated a mutant in *S. aureus* strain Newman that lacked both the genes for Ecb and Efb. In contrast to the wild-type strain, this ΔEcbΔEfb mutant was unable to grow in human whole blood, caused lower lethality in a mouse intravenous infection model and was cleared more easily in a mice pneumonia model. Intranasal infection of mice with the ΔEcbΔEfb mutant strain resulted in the recruitment of more neutrophils towards the lungs compared to an infection with wild type Newman, indicating that

Ecb and Efb expression is crucial for inhibition of C5a generation and neutrophil influx *in vivo* as well. However, mice are not natural hosts to *S. aureus* and mice do not always harbor antibodies against *S. aureus* resulting in different complement activation as compared to the human host where infection with *S. aureus* leads to activation of the CP via binding of IgG to the surface[16]. Moreover, mice are very resistant against *S. aureus* infections so high amounts of bacteria are needed to establish an infection[17]. On the other hand, mice models can give more information than *in vitro* data since proteins of interest need to be produced during the actual infection and all immune cells are present in contrast to *in vitro* situations where we normally use only one cell type. In these *in vivo* models, all immune cells will respond to the infection in their natural way, making these mice models a valuable addition to our *in vitro* molecular mechanistic data.

Importance of immune evasion clusters

Genes encoding SCIN, Efb and Ecb are all present on immune evasion clusters (IEC) together with other known immune evasion molecules. IEC-1 is present on a prophage and harbors the genes coding for SCIN, Chemotaxis inhibitory protein of *S. aureus* (CHIPS), Staphylokinase (SAK) and Staphylococcal entrotoxin A (SEA)[18]. CHIPS binds to the C5a receptor and the formylated peptide receptor thereby inhibiting neutrophil migration towards C5a and fMLP[19], SAK activates human plasminogen on the bacterial surface thereby removing C3b and IgG from the target surface preventing phagocytosis[20]. SEA is a superantigen but also has the capacities to modulate the function of several chemokine receptors[21]. Efb and Ecb are located on IEC-2, which contains genes for formyl peptide receptor like-1 Inhibitory Protein (FLIPr)/FLIPr-like, SCIN-B/SCIN-C, α-toxin and Staphylococcal superantigen-like (SSL) 11,12 and 13[15,22,23]. Next to this, other SSLs, with additional immune evasion functions[24-26], are present on pathogenicity island (SaPI) 2[27].

It is striking that most of the genes encoding immune modulating proteins are present on IECs or SaPIs, indicating that the presence of these genes on clusters facilitates their spread among *S. aureus* strains. It is known that IEC-1 is a very dynamic DNA element which is shown to be easily transferred among *S. aureus* strains by a diverse group of β-hemolysin-converting bacteriophages [18]. IEC-2 is present in all sequenced *S. aureus* strains and in 91 clinical isolates[15]. Moreover, the presence of transposases and bacteriophage remnants suggests that is probably used to be a mobile element. Recently, it was shown that among others, genes encoding complement evasion molecules are transcribed in operons. It was determined that Efb and SCIN-B, both present on IEC-2, are transcribed in one operon and that they have a synergistic effect on complement inhibition[28]. Yet another reason for *S. aureus* to exchange their immune evasion molecules in clusters might be to preserve operon structures.

A surplus of complement evasion molecules; luxury or necessity, from a bacterial point of view

To date we know that *S. aureus* carries the genes coding for at least a dozen complement inhibitory proteins that all have unique functional properties and block the complement system at different steps (See Figure 2). It is likely that all these molecules are produced in the human host since they are present in all human *S. aureus* clinical isolates and all healthy individuals have antibodies against all these proteins[7,8,15]. For Ecb and Efb we demonstrated that they are expressed *in vivo* and contribute to the pathogenesis of *S. aureus* infections (Chapter 7). The production of so many complement inhibitory proteins seems energy consuming. However since most of them

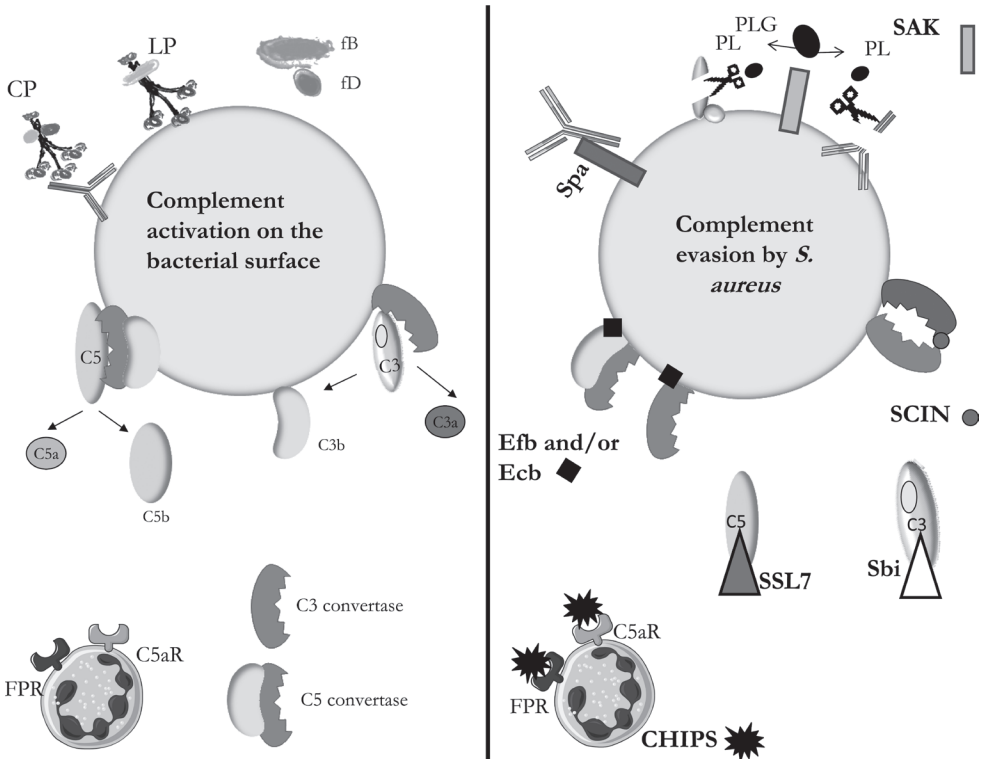

Figure 2: Complement evasion by *S. aureus*
Left, complement activation on the bacterial surface. Complement is activated via the CP, the LP or the AP. All pathways recognize different activation markers but all convert in the formation of C3 convertases that cleave C3 into C3a and C3b. C3b will be deposited on the bacterial surface which will enhance phagocytosis and is needed to form C5 convertases. C5 convertases cleave C5 into the strong chemoattractant C5a (recognized by neutrophils) and C5b. *Right,* complement evasion by *S. aureus*. Activation of the CP is inhibited by staphylococcal protein A (SpA) which binds the Fc region of IgG molecules[50]. Staphylokinase (SAK) targets plasminogen (PLG) to the surface and activates it into plasmin (PL). In turn, PL cleaves C3b and IgG, preventing phagocytosis[20]. SCIN forms dimeric C3 convertases thereby inhibiting C3b deposition and phagocytosis[8-10], Efb and Ecb bind to the C3d domain of C3b-containing convertases inhibiting the C3 convertase of the AP and the C5 convertase of all pathways[15]. SSL7 binds C5 preventing its cleavage[26] and Sbi activates C3[47]. Furthermore, CHIPS binds to the C5aR and the FPR thereby preventing neutrophil migration towards C5a and fMLP[19].

attack the complement system at a different target, it demonstrates a smart evolutionary strategy and reveals the efficacy of our own immune system. Moreover, other microbes have developed other mechanisms to avoid the attack of the human immune system also involving numerous genes. A generally accepted mechanism to evade the human complement system is the expression of a thick capsule for example by gram-negative bacteria such as *Neisseria meningitides* and gram-positive bacteria such as Group A *Streptococcus*[29,30]. A bacterium needs to express at least 15 genes to produce such a thick capsule. Moreover, herpes viruses, which have a very small genome, are known to express several immune evasion molecules to combat the host-mediated immunity. They encode several molecules that all attack important steps involved in antigen processing[31,32].

The presence of this large arsenal of immune evasion molecules makes the bacterium ready to combat the immune system of different human individuals which all harbor a different variety of antigens and genomic variation in their immune response. Furthermore, the bacterium can express different molecules during different growth stages. Next, it is shown that most of these relatively small immune evasion molecules are multi-functional as well. SSL-5 was shown to hamper neutrophil rolling and adhesion to endothelial cells by binding to the leukocyte adhesion molecules P-selectin glycoprotein ligand-1, but the protein also inhibits activation of G-coupled receptors[24,25]. Another duo function was shown for Efb, which can bind fibrinogen and thereby modulate platelet activation, and on the other hand bind C3b and inhibit complement activation and subsequent neutrophil migration[15,33]. It is likely that other complement evasion molecules fulfill dual functions that we are not aware of at this moment. SCIN-B and SCIN-C are highly human specific in their action of complement inhibition, yet they are present in *S. aureus* strains isolated from animals which makes it likely that SCIN-B and SCIN-C have an unknown function in animals.

Despite the fact that *S. aureus* produces a large arsenal of immune evasion molecules healthy humans most frequently win the battle. This tells us something about the efficacy of our own immune system. Next to the complement system we have several other mechanisms to fight bacteria and other microorganisms. The cells of the innate immune system are aggressive and can kill and remove invading microbes within minutes[16], probably before the immune evasion molecules are produced. Microbes are recognized by human cells via pattern recognition receptors that recognize specific patterns on the target surface. Immune cells can for instance detect lipopolysaccharides via Toll-like receptor 4, or formylated peptides that are secreted by all bacteria via the FPR. Next, antibodies produced by the adaptive immune response can neutralize bacterial components including toxins, superantigens but also immune evasion molecules. In conclusion, the struggle of the fittest seems currently won by the human immune response but bacteria are catching up chattering immune evasion molecules and antibiotic resistance very fast and efficient as shown for the current epidemic community acquired *S. aureus* strain USA300.

Therapeutic implications

The complement system plays a role in a wide range of autoimmune and chronic inflammatory diseases among which rheumatoid arthritis[34,35]. Since the proteins described in this thesis effectively inhibit the complement system they become attractive targets for novel anti-inflammatory therapies. Some proteins from bacterial origin are already used as therapeutic proteins. Streptokinase and staphylokinase are for instance used to treat thrombolysis in acute myocardial infarction[36,37]. Such therapies educated us on the disadvantages of bacterial derived proteins as therapeutics. Administration of Staphylokinase and Streptokinase induced antibody formation in the majority of the patients. These antibodies turned out to be neutralizing which resulted in loss of function of the drug meaning that higher amounts of drugs need to be administered to overcome this loss of function. Extensive research on Staphylokinase shows that variants with a decreased antibody binding also induced less antibody production in the human body[38]. It is already shown that SCIN, Efb and Ecb are immunogenic since all healthy volunteers tested so far have antibodies against these proteins[8,15] and this will limit their use for anti-inflammatory drugs. On the other hand, structures and functions of these proteins are extensively studied which maybe opens the possibility to design small, less immunogenic variants of these proteins.

Although Staphylococcal disease was already reported in 1882[39], lately the emergence of meticillin-resistant strains (MRSA) in the hospital makes the bacterium a real public threat[40]. An even bigger health problem is the emergence of the community-associated MRSA (CA-MRSA). CA-MRSA strains cause infections in healthy individuals without predisposing risk factors and outside of the hospital. CA-MRSA strain USA300 has become an epidemic strain in the USA. USA300 isolates were initially only resistant to semi-synthetic penicillins and macrolides. Over the last five years the strain has become a highly resistant strain, harboring resistance to clindamycin, tetracyclin and mupirocin. Next, isolates are found that are resistant against fluoroquinolones and vancomycin[41,42]. New antimicrobial agents are under investigation, but because of this multidrug resistance it would be likely that *S. aureus* will gain resistance against these drugs as well[43]. Therefore, the urge for vaccination is raising[41,42].

Currently, despite several clinical phase 3 trials[44,45], no good vaccine against *S. aureus* is available yet. Many different and difficult questions need to be answered before we are able to make a good vaccine against *S. aureus*. First of all, we need to consider who the vaccine is for. People that are at high risk to develop *S. aureus* infections are for instance immunocompromised people, but a vaccine for this patient group should be a passive immunization. On the other hand, since CA-MRSA is an epidemic nowadays we could consider developing a vaccine for the healthy human population as well, but that will include an active immunization strategy rather than a passive strategy[43]. The capsule of *S. aureus* is an obvious target, since successful vaccines against capsules of other pathogens are already introduced[46]. A vaccine against capsular polysaccharides serotype 5 (CP5) or serotype 8 (CP8) was raised but despite successful experiments with mice and rats a phase 3 trial among hemodialysis patients showed no protection[43]. An example of passive immunization approaches is the administration of pooled human immunoglobulin preparation from donors with high antibody titers against Clumping factor A (ClfA). Although the phase 2 trial showed protective responses against *S. aureus* in infants, the phase 3 trial showed no significant effects[44].

We now know that a vaccine solely targeting capsule is not effective, also a vaccine composed of antibodies directed against ClfA did not turn out to give protection against *S. aureus*. An effective vaccine against *S. aureus* should probably be directed against the capsule, some proteins that are important for adherence to human cells and some immune evasion molecules. Antibodies directed against the capsule can direct the complement activation towards the bacterial surface, but if neutralizing antibodies are generated against proteins that are important for adherence we are maybe able to inhibit adhesion of *S. aureus* towards for instance endothelial cells, thereby maybe preventing colonization. Next, if neutralizing antibodies are directed against immune evasion molecules they can hamper their function, which will make the bacterium less virulent. It is already known that monoclonal antibodies directed against SCIN can be neutralizing[8]. We and others showed that Ecb, Efb and Staphylococcus aureus binder of IgG (Sbi) are able to prevent binding of C3d to CR2 thereby inhibiting B-cell proliferation[47,48](Chapter 5). Furthermore, our own *in vivo* models (Chapter 7) indicate strongly that the removal of Ecb and Efb results in more efficient removal of the bacterium. Therefore, it would be important to incorporate immune evasion proteins in a vaccine as well. Secretion of these proteins by *S. aureus* will result in inhibition of the immune system. Therefore, inhibiting the inhibitor by inducing neutralizing antibodies through vaccination could prove a novel and useful strategy to improve vaccine efficacy.

Conclusion

The human immune system uses a diverse array of molecules to sense invading microorganisms. Here, I showed that the mechanism of action for different bacterial proteins that tackle the immune system can be equally redundant. Also, the insight in the molecular mechanism of immune evasion, provides better insight on the function of the different elements of our own immune system. Furthermore, these mechanisms can be crucial in the development of new anti-inflammatory drugs or vaccines against *S. aureus*. In this thesis I was able to show the relevance of expression of immune evasion molecules for the pathogenesis of *S. aureus*.

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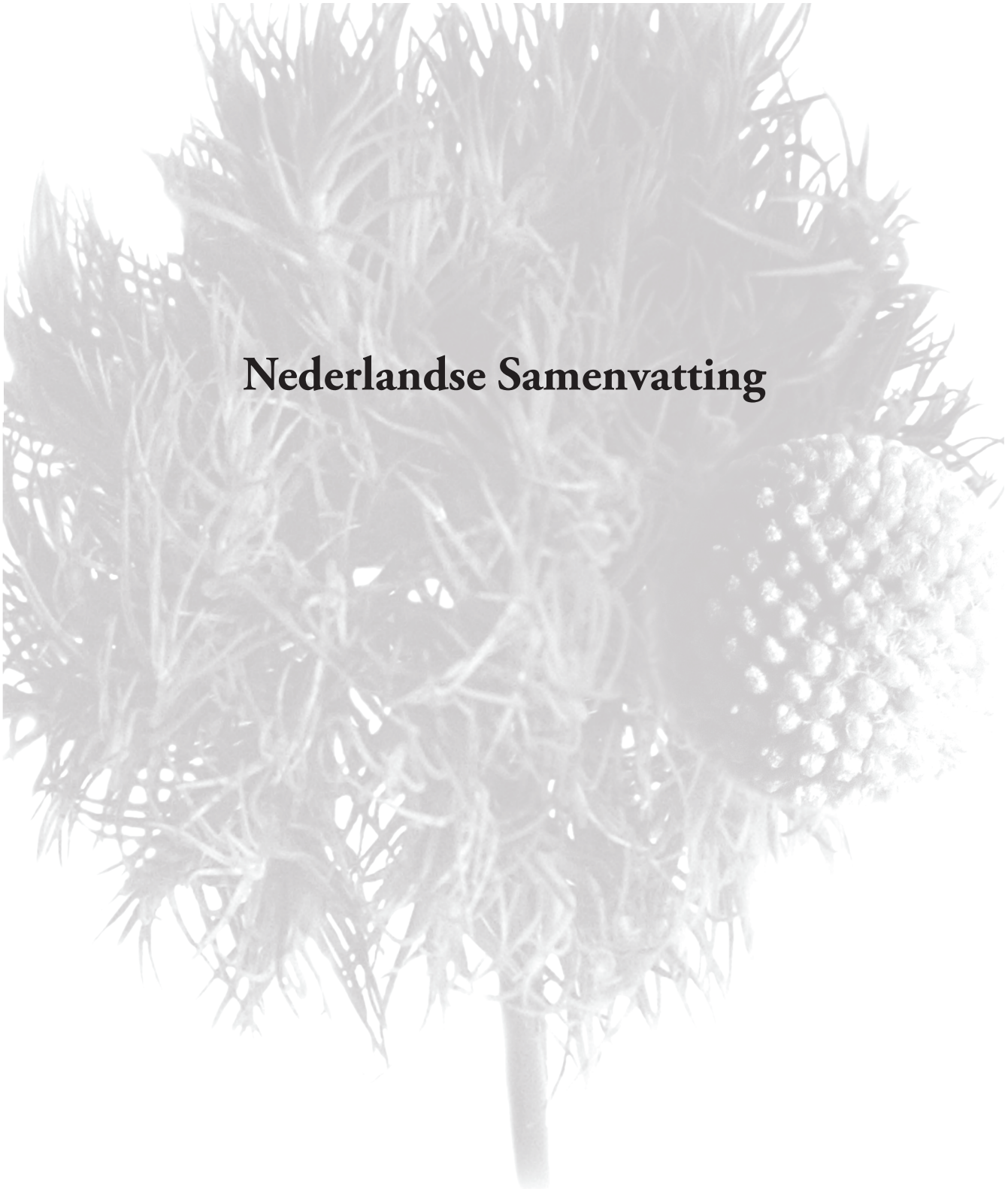

Nederlandse Samenvatting

Inleiding

Het immuunsysteem beschermt ons tegen ziekmakende bacteriën, virussen en schimmels. Als een van deze organismen het menselijk lichaam binnendringt wordt het immuun systeem geactiveerd waarop de binnendringer wordt opgeruimd. Het immuunsysteem bestaat uit het "aangeboren" (innate) immuunsysteem en het "aangepaste" (adaptive) immuunsysteem. Het aangeboren immuunsysteem werkt binnen enkele seconden omdat het bacteriën en andere micro-organismen herkend aan bepaalde geconserveerde structuren die het menselijk lichaam niet bevat. Het aangepaste immuunsysteem werkt pas na ongeveer een week.

Het aangeboren immuunsysteem bestaat uit een cellulaire en humorale (serum) component die beiden in staat zijn bacteriën te herkennen en op te ruimen. De cellulaire kant bestaat uit neutrofielen, monocyten en macrophagen. Deze cellen zijn uitgerust met verschillende receptoren die bacteriën en andere micro-organisme herkennen, daarnaast zijn ze agressief en in staat bacteriën op te eten (phagocyteren) en te doden. Cellen herkennen bacteriën beter als ze beladen zijn met specifieke moleculen (opsonins), deze moleculen worden op de bacterie gezet door de humorale kant van het aangeboren immuunsysteem. De humorale kant van het immuunsysteem (het complementsysteem) bestaat uit 30 eiwitten die zich in het serum of op cellen bevinden. Het complementsysteem bestaat uit drie verschillende routes: de klassieke route, de lectin route en de alternatieve route. Alle drie de routes worden geactiveerd via andere activatoren, maar ze komen allemaal samen in de belangrijkste stap van het complement systeem, het knippen van C3. Het knippen van C3 gebeurd door gevormde C3 convertases en leidt tot formatie van C3b. C3b wordt afgezet op de bacterie en wordt herkend door immuun cellen die bacteriën geopsonizeerd met C3b zullen phagocyteren en doden. Daarnaast is C3b belangrijk voor de volgende stap in het complement systeem de formatie van C5 convertases. C5 convertases knippen complement component C5 wat resulteert in de formation van C5a en C5b. C5a is belangrijk voor de migratie van immuun cellen naar de plek van de infectie, C5b is belangrijk omdat het nodig is voor de formatie van C5b-9 (het membrane attack complex) wat Gram-negatieve bacteriën in enkele minuten lyseren en daardoor doden.

Staphylococcus aureus

De bacterie *Staphylococcus aureus* (*S. aureus*) is een Gram-positieve bacterie die bij ongeveer 40% van de mensen voorkomt op de huis of op de slijmvliezen. Meestal zorgt deze bacterie niet voor problemen bij de dragen (hooguit het ontstaan van "jeugd" puistjes), maar in sommige gevallen kan deze bacterie een breed scala aan ziektes vooroorzaken. Als de bacterie ons lichaam binnendringt, wat meestal gebeurd bij open wonden of na operaties, kan dit leiden tot huidinfecties, maar ook ernstige ziektes zoals voedselvergiftiging, endocarditis en septische shock. Zoals hierboven beschreven zou het immuunsysteem zonder problemen deze bacterie moeten herkennen en opruimen, maar dit blijkt niet altijd het geval te zijn. *S. aureus* heeft zich aangepast aan het immuun systeem en kan verschillende eiwitten uitscheiden die ervoor zorgen dat het immuun systeem minder effectief is in het opruimen van deze bacterie. Een belangrijk voorbeeld van een eiwit dat *S. aureus* produceert om het immuunsysteem te ontwijken is CHIPS

(Chemotaxis inhibitory protein of *Staphylococcus aureus*). CHIPS bindt aan twee receptoren op neutrophielen (de C5a Receptor en de formylated peptide receptor) die er normaal gesproken voor zorgen dat de neurtophiel migreert naar de plek van infectie. Door aan deze receptoren te binden zorgt CHIPS ervoor dat neutrophielen op afstand worden gehouden wat er voor zorgt dat *S. aureus* niet kan worden gedood. Naast CHIPS produceert *S. aureus* nog vele andere moleculen die er op een of andere manier voor zorgen dat het immuun systeem niet adequaat reageert.

In dit proefschrift worden de immuun modulerende eigenschappen van staphylococcal eiwitten bestudeerd. Er is gekeken naar het mechanisme van Extracellular fibrinogen binding protein (Efb), Extracellular complement binding protein (Ecb) en naar Staphylococcal complemenent inhibitor (SCIN). Al hoewel deze eiwitten qua grootte en structuur heel erg op elkaar lijken, blijkt hun mechanisme compleet verschillend. Daarnaast wordt er voor de eerste keer naar de functie van deze eiwitten in diermodellen gekeken.

SCIN bindt aan C3 convertases van alle complement activatie routes en zorgt ervoor dat phagocytose van de bacterie geremd wordt omdat er minder C3b op de bacterie wordt geplaatst. Daarnaast zorgt SCIN er ook voor dat er geen C5a generatie plaatsvindt, wat ervoor dat neutrofielen niet migreren naar de plaats van infectie. Recentelijk is gevonden dat SCIN C3 convertases stabiliseert en twee convertases aan elkaar kan koppelen waardoor dimeren ontstaan. Deze stabilizatie van convertases op de bacterie lijkt in eerste instantie niet verstandig omdat C3b moleculen (die herkend worden door neutrophielen) die in deze convertases zitten ook op de bacterie blijven zitten. In **hoofdstuk 2** laten we zien, dat het vormen van dimeren juist van essentieel belang is voor het remmen van het immuunsysteem. We hebben laten zien dat het C3b moleculen die in convertases dimeren zitten niet meer kunnen worden herkend door de receptoren op neutrophielen en anderen immuun cellen.

In **hoofdstuk 3** beschrijven we de ontdekking van een nieuw cluster met eiwitten die allemaal iets doen met het immuunsysteem. We beschrijven twee SCIN homologen, te weten SCIN-B en SCIN-C, die dezelfde functie als SCIN blijken te hebben. Daarnaast beschrijven we Efb en Ecb. Beiden eiwitten zijn homologen van elkaar en binden aan het C3d, C3b, iC3b en C3. We laten zien dat Efb en Ecb specifiek convertases remmen die een C3b molecuul bevatten, te weten het C3 convertase van de alternatieve route en de C5 convertases van alle complement activatie routes. Beiden zijn erg effectief in het remmen van C5a generatie, wat ervoor zorgt dat neutrofielen niet naar de infectie haard zullen migreren. Verder bleek dat Efb en Ecb niet alleen het menselijk immuunsysteem remmen maar ook het immuunsysteem van verschillende dieren. Hierdoor werden voor het eerst deze kleine immuun ontwijkende eiwitten getest in een diermodel. Pre-incubatie van muizen met recombinant Ecb zorgde voor complete remming van neutrofiel migratie naar de plek waar de ontsteking plaatsvond.

In **hoofdstuk 4** wordt het exacte mechanisme van Ecb beschreven. Het C3d bindende domain van Ecb bleek van essentieel belang voor de inhibitie van C5 convertases. Daarnaast stabiliseert Ecb pro-convertases (C3bB) wat tot gevolg heeft dat de actieve convertases (C3bBb) niet worden gevormd.

In **hoofdstuk 5** blijkt dat Ecb niet alleen het aangeboren immuunsysteem kan remmen maar ook het aangepaste. Eerder is al beschreven dat Ecb aan C3d, een domain in het C3 molecuul, kan binden. C3d is erg belangrijk voor de activatie van het aangepaste immuunsysteem. Tijdens activatie van het aangepaste immuunsysteem worden B-cellen geactiveerd door antigenen, deze acitvatie gaat 1000 keer efficiënter in de aanwezigheid van C3d. Hier laten we zien dat binding van Ecb aan C3d ervoor zorgt dat C3d niet meer aan B-cellen kan binden. Dit zal hoogstwaarschijnlijk leiden tot remming van het aangeboren immuunsysteem.

In **hoofdstuk 6** wordt de functie van het fibrinogeen bindende deel van Efb onderzocht. In eerste instantie leken Efb en Ecb eenzelfde functie te hebben, beiden remmen ze C5a generatie volledig. In tegenstelling tot Ecb kan Efb niet alleen aan C3 eiwitten binden maar ook aan fibrinogeen. Hier laten we zien dat binding van Efb aan fibrinogeen een belangrijke extra functie heeft ten opzichte van Ecb. Efb is in staat op C3b binding aan neutrofielen te blokkeren, daarnaast is Efb in staat om phagocytose van bacteriën te remmen in aanwezigheid van fibrinogeen terwijl Ecb daartoe niet instaat is.

In **hoofdstuk 7** hebben we voor de eerste keer laten zien dat het uitscheiden van kleine immuun modulerende eiwitten van groot belang is voor de virulentie van *S. aureus*. In twee muismodellen blijkt dat een *S .aureus* stam die geen Efb en Ecb kan maken veel minder virulent is in vergelijking tot een wild type *S. aureus* stam. Muizen die geïnfecteerd werden met de wild type stam overleefde de *S. aureus* infectie niet terwijl muizen die geïnfecteerd werden met een *S. aureus* stam die geen Efb en Ecb tot expressie bracht wel bleven leven. Daarnaast zat er een groot verschil tussen de hoeveelheid abscessen en de hoeveelheid bacteriën in de nieren. Ook in een pneumonea model is te zien dat muizen die geinocculeerd worden met wild type bacteriën meer bacteriën in hun longen hebben dan muizen die geinocculeerd worden met een gemuteerde stam dit is waarschijnlijk te wijten aan het feit dat er veel meer neutrofielen aanwezig zijn in de longen van muizen die geïnfecteerd zijn met de bacterie die geen Efb en Ecb meer maakt.

Conclusie

Het humane immuunsysteem gebruikt een grote hoeveelheid moleculen die binnendringende bacteriën herkennen. In dit proefschrift heb ik verschillende eiwitten beschreven die *S. aureus* uitscheidt om ons efficiënte immuunsysteem te ontwijken. Het onderzoeken van deze eiwitten kan van groot belang zijn voor het ontwikkelen van ontstekingremmende medicijnen of een vaccinatie tegen *S. aureus.* Verder heb ik laten zien dat het uitscheiden van deze kleine moleculen van levensbelang is voor de bacterie omdat hij zonder expressie van deze moleculen niet overleefd in de muis en hoogstwaarschijnlijk ook niet in de mens.

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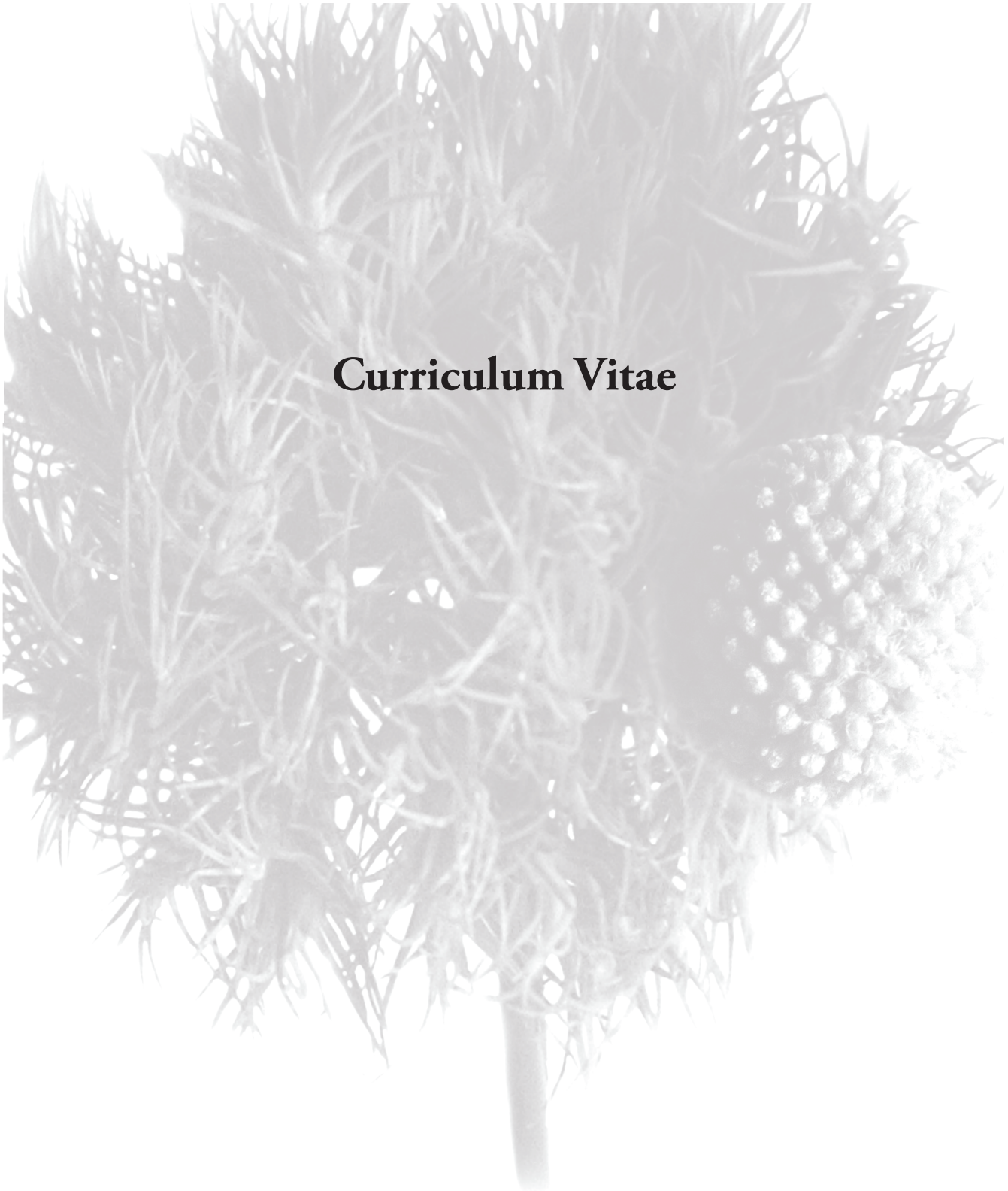
Ilse



Curriculum Vitae

Ilse Jongerius was born on December 30, 1981 in Gouda, The Netherlands. She grew up in Oudewater and obtained here VWO-Athenaeum diploma at high school "Minkema College" in Woerden, The Netherlands in 2000. In the same year she started her study Biology at the University of Utrecht. During this study she performed her first internship under supervision of Dr. M.P. Bos and Prof. dr. J.P.M. Tommassen at the department of Microbiology at the University Utrecht. Her second internship was performed under supervision of Dr. P.J.A. Haas at the department of Medical Microbiology at the University Medical Center Utrecht, after which she graduated in 2005.

In September 2005 she started her PhD training at the department of Medical Microbiology under supervision of Prof. dr. J.A.G. van Strijp and Dr. S.H.M. Rooijakkers. The results of her PhD training are described in this thesis.

In the summer of 2009 the author will pursue her post-doctoral career at Imperial College Londen where she will examine the complement mediated interaction between *Neisseria meningitidis* and epithelial cells.

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(Ilse_jongerius@hotmail.com)

List of Publications

Ilse Jongerius, Jörg Köhl, Manoj. K. Pandey, Maartje Ruyken, Kok P.M. van Kessel, Jos A.G. van Strijp and Suzan H.M. Rooijakkers. *Staphylococcal complement evasion by various convertase-blocking molecules*. J. Exp. Med, 2007 Okt 1

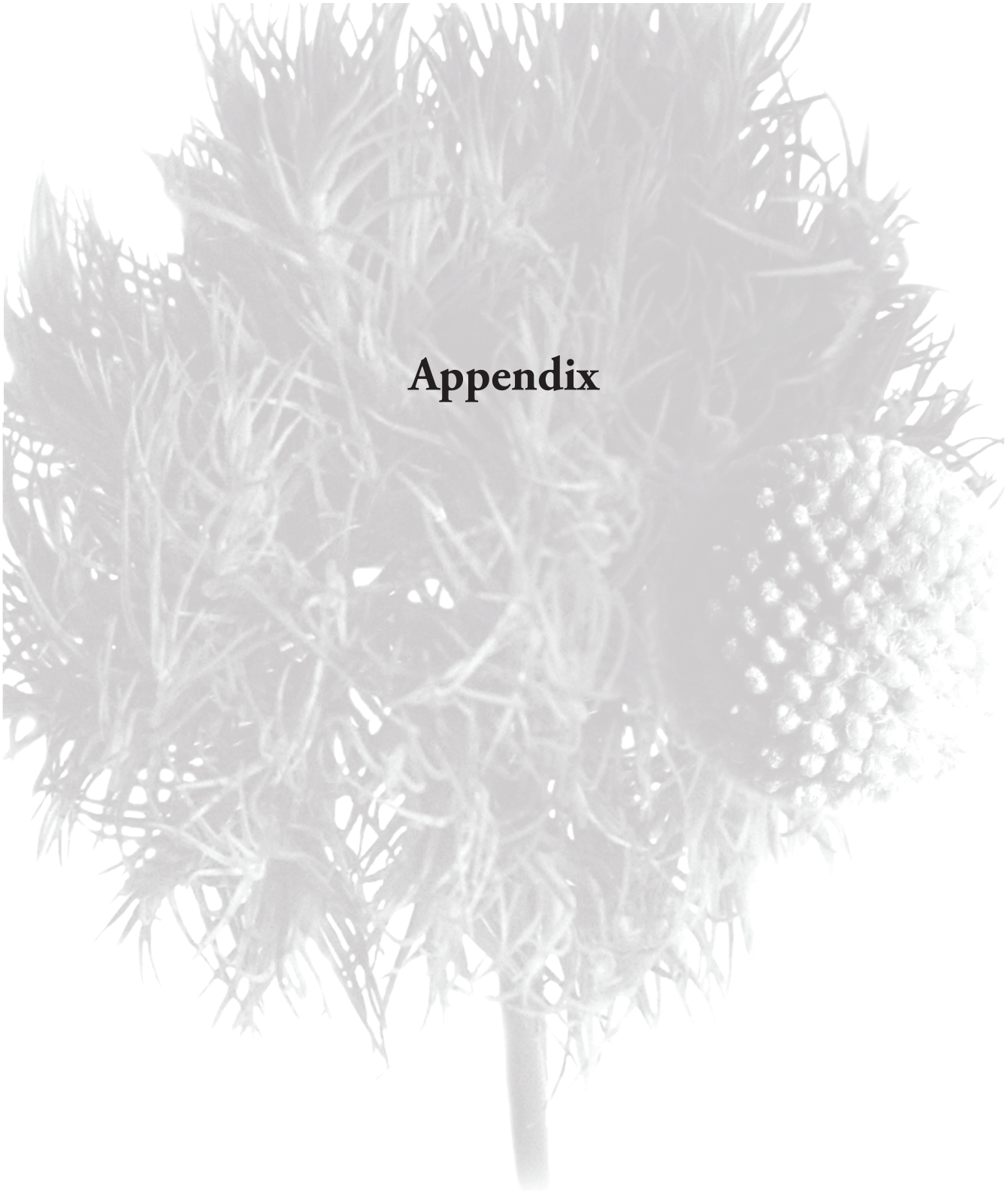
Ilse Jongerius, Mannon Puister, Jin Wu, Maartje Ruyken, Jos van Strijp and Suzan Rooijakkers. *Immunomodulatory functions of convertase dimerziation by S. aureus complement inhibitor*. J.Immunol, 2010, Jan 1.

Ilse Jongerius, Maartje Ruyken, Jos van Strijp and Suzan Rooijakkers. *Extracellular complement binding protein inhibits the formation of the catalytic subunits of convertases: C2a and Bb.* In press, 2010

Nicole ten Broeke-Smits, Tessa Pronk, **Ilse Jongerius**, Oskar Bruning, Floyd Wittink, Timo Breit, Jos van Strijp, Ad Fluit, Edwin Boel. *Operon Structure of Staphylococcus aureus.* Nucl. Acids Res, 2010, Feb. 16

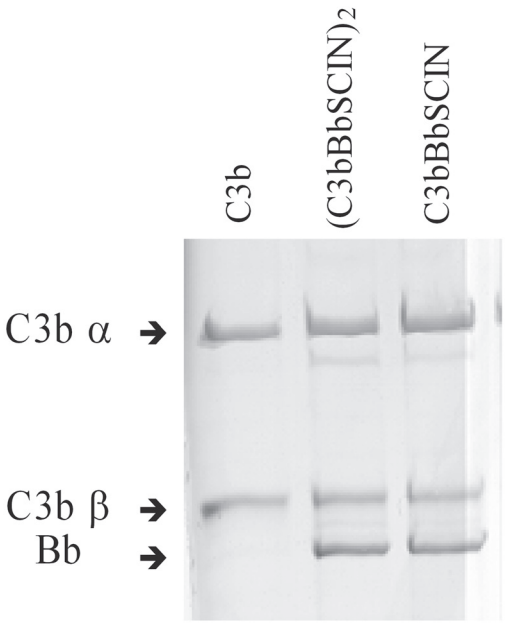
Book Chapters

Ilse Jongerius, Sanjay Ram and Suzan Rooijakkers. *Bacterial complement escape*. Chapter of: Pathogen-Derived Immunomodulatory Molecules. ISBN: 978-1-58706-334-3 publication date: august 15, 2009.



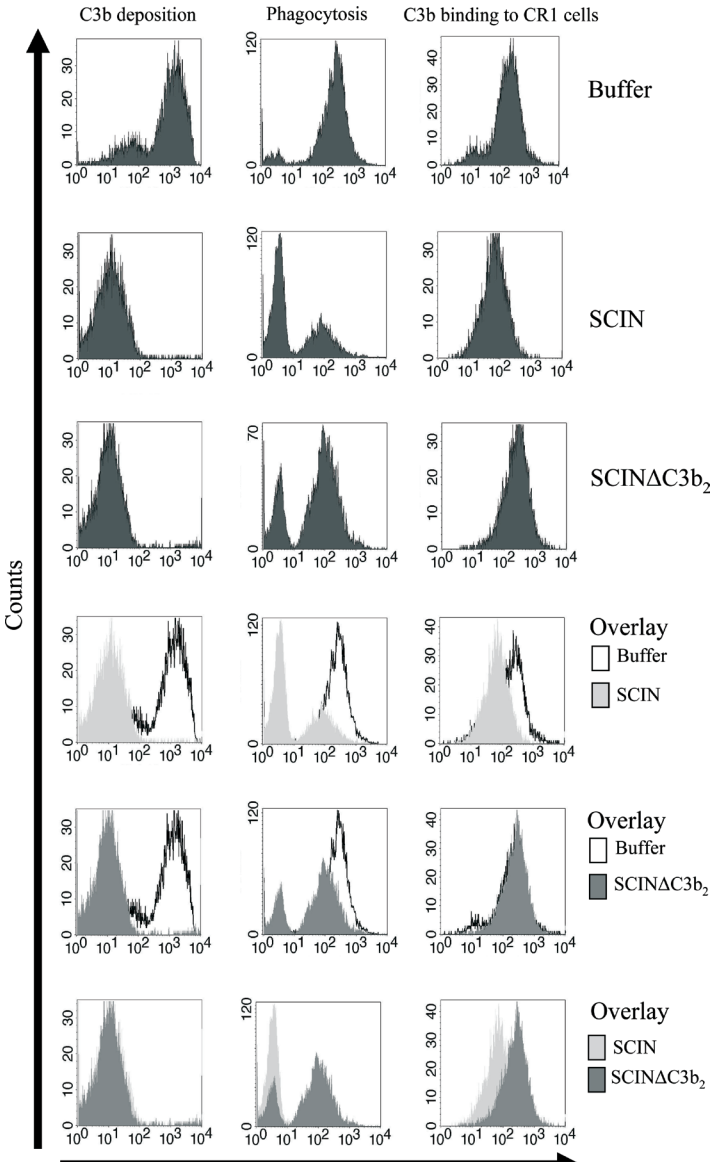
Appendix

SUPPLEMENTARY FIGURE 1, CHAPTER 2



Supplementary figure 1: Quantification of C3b molecules in purified SCIN-convertases
The concentration of C3b molecules present in purified SCIN- or SCINΔC3b$_2$-convertases was analyzed by SDS-PAGE and silverstaining. Representative for all experiments performed with purified convertases.

SUPPLEMENTARY FIGURE 2, CHAPTER 2



Supplementary figure 2: Flow cytometry histograms.
Representative histograms of flow cytometry data obtained for C3b deposition, phagocytosis and C3b binding to CRO-CR1 cells (similar to histograms obtained for PMN and Jurkat-CRIg cells).

SUPPLEMENTARY FIGURE 1, CHAPTER 3

A

```
SCIN    ST--SLPTSNEYQNEKLANELKSLLDELNVNELATGSLNTYYKRTIKISGQKAMYALKSK 58
SCIN-B  SSLDKYLTESQFHDKRIAEELRTLLNKSNVYALAAGSLNPYYKRTIMMNEYRAKAALKKN 60
SCIN-C  SSKKDYIIQSEFHDKRIAEELKSLLDQSYVNDLAAGSLNPYYKRMIMMNQYRAKAALKSN 60
ORF-D   SK--SETTSHTYQHQALVDQLHELIANTDLNKLSHLNLDAYQKRDILAAHYIAKSAIRTK 58
        *.  .   .  ::.: :.::*: *: :  :  *:  .*:.* ** *      *  *::.:

SCIN    DFKKMSEAKYQLQKIYNEIDEALKSKY- 85
SCIN-B  DFVSMADAKVALEKIYKEIDEIINR--- 85
SCIN-C  NFAKMAEAKVGLENIYKEIDEIINR--- 85
ORF-D   NLDQMTKAKHRLESIYDSISNPLHSQNN 86
        :: .*:.**  *:.**..*.: ::
```

B

```
Efb     SEGYGPREKKPVSINHNIVEYNDGTFKYQSRPKFNSTPKYIKFKHDYNILEFNDGTFEYG 60
Efb-C   ------------------------------------------------------------
Ecb     ------------------------------------------------------QTKNVE 6


Efb     ARPQFNKPAAKTDATIKKEQKLIQAQNLVREFEKTHTVSAHRKAQKAVNLVSFEYKVKKM 120
Efb-C   ----FNKPAAKTDATIKKEQKLIQAQNLVREFEKTHTVSAHRKAQKAVNLVSFEYKVKKM 56
Ecb     AAKKYDQYQTNFKKQVNK--KVVDAQKAVNLFKRTRTVATHRKAQRAVNLIHFQHSYEKK 64
        ::: :: . ::* *:::**: *. *::*:**::*****:****: *:: .:*

Efb     VLQERIDNVLKQGLVR 136
Efb-C   VLQERIDNVLKQGLVR 72
Ecb     KLQRQIDLVLKYNTLK 80
       **.:** *** . ::
```

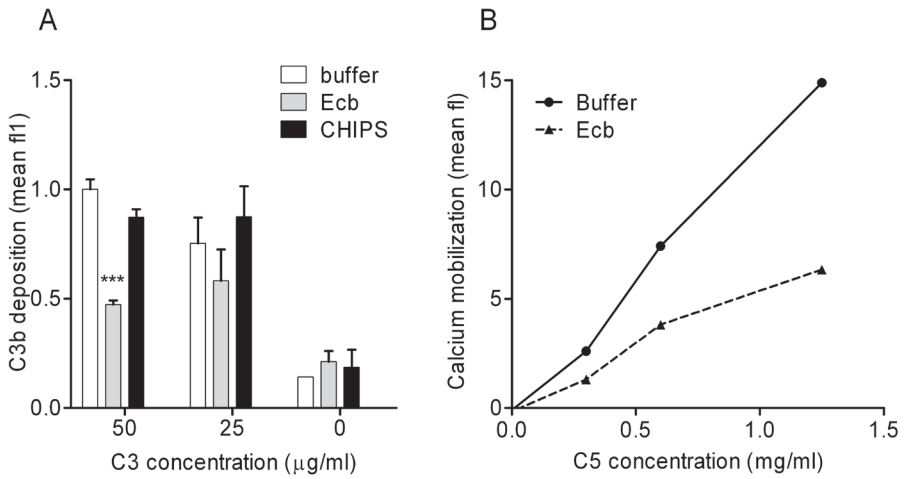
Figure S1.
(**a**) Sequence alignment of three *S. aureus* open reading frames which are highly homologous to SCIN. SCIN-B (48% homology), SCIN-C (46%) and ORF-D (33%) are excreted proteins with a MW of 10 kD. (**b**) Sequence alignment of Efb, Efb-C and Ecb. The N-terminus (aa 1-64) of Efb mediates binding to fibrinogen while the C-terminus (Efb-C, aa 65-136) binds the C3d domain of C3 and C3b. Sequences of exported proteins are presented. '*' indicate identical residues, ':'indicate conserved substitutions and '.' indicate semi-conserved substitutions (CLUSTAL W (1.83)).

SUPPLEMENTARY TABLE 1, CHAPTER 3

protein	Primer name	Primer sequence
SCIN-B	SCIN-B For	5'-GAGTAGTCTGGACAAATATTTA-3'
	SCIN-B ForI	5'- CTGGACAAATATTTAACTGAAAGTC-3'
	SCIN-B Rev	5'-GGAATTCCTTATCTATTTATAA-3'
	SCIN-B RevI	5'- GATACGAAATCATTTT TCTTA-3'
SCIN-C	SCIN-C For	5'-GAGTAGTAAGAAAGACTATAT-3'
	SCIN-C ForI	5'- AAGAAAGACTATATAATTCAAAGTG- 3'
	SCIN-C Rev	5'-GGAATTCCTTATCTATTTATAATTTCA-3'
	SCIN-C RevI	5'-TTTGCGAAATTATTACTTTTT-3'
ORF-D	ORF-D For	5'-GAGCAAATCTGAAACTACATCACAT-3'
	ORF-D Rev	5'-GGAATTCCTTAATTGTTTTGTGAATGC-3'
Ecb	EKEcb Rev	5'-TAGCAGCTTCAACGTTTTTAGTTTGCAAACTAAAAACG TTGAAGC-3'
	EKEcb For	5'-GGATCTGTACGACGATGACGACAAGCAAACTAAAAACG TTGAAGC-3'
	Efblike Rev	5'- GGAATTCCTTATTTTAAAGTATTAT-3'
Efb	EKEfb Rev EKEfb For	5'- ACCGTATCCTTCGCTCTTGTCGTCATCGTCGTACAG-3'
	Efb Rev	5'-GACGATGACGACAAGAGCGAAGGATACGGTCCAAGA-3'
		5'- *G*GAATTCCTTATCTCACTAATCCTTGTTTTAATAC-3'
Efb-C	EKEfb-C Rev	5'- TGCTGGTTTATTAAACTTGTCGTCATCGTCGTACAG-3'
	EKEfb-C For	5'-GACGATGACGACAAGTTTAATAAACCAGCAGCGAAA-3'
	Efb-C Rev	5'- GGAATTCCTTATCTCACTAATCCTTGTTTTAATAC-3'

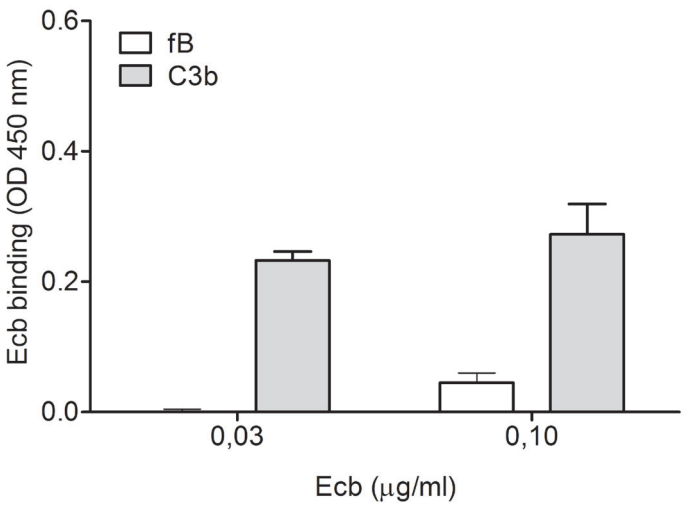
[T]Table I. Primers used in this study.

SUPPLEMENTARY FIGURE 1, CHAPTER 4



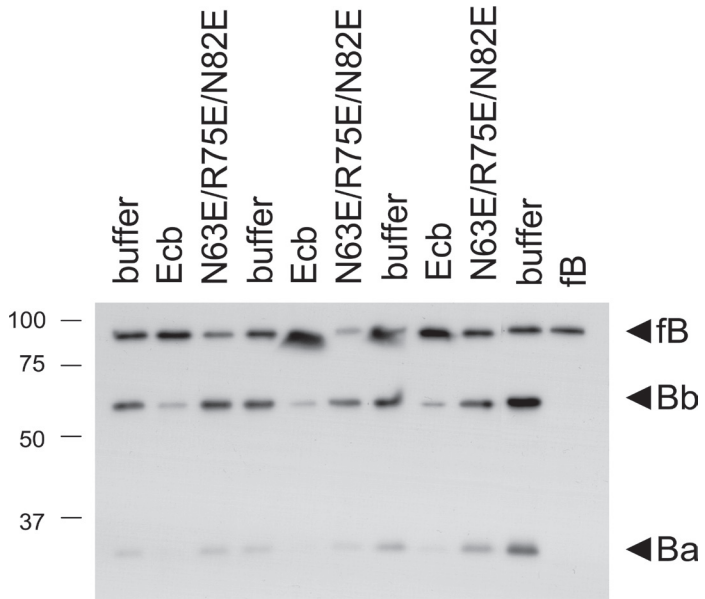
Supplementary Figure 1: AP activation in the presence of properdin
(a) C3b deposition via the AP in the presence of properdin. Pre-opsonized bacteria were incubated with fB, fD, properdin and C3 in the presence or absence of Ecb (10 μg/ml). (b) C5a generation via the AP in the presence of properdin. Opsonized bacteria were incubated with fB, fD, properdin, C3 and C5 in the presence of Ecb (10 μg/ml). C5a generation in the supernatant was tested by calcium mobilization. Figure a represents the mean ± SEM of three separate experiments, figure b is a representative of three separate experiments.

SUPPLEMENTARY FIGURE 2, CHAPTER 4



Supplementary Figure 2: Ecb binding to immobilized C3b or fH.
The figure represents the mean ±SEM of 3 separate experiments.

SUPPLEMENTARY FIGURE 3, CHAPTER 4



Supplementary Figure 3: Fluid-phase fB conversion
Fluid-phase fB conversion. C3b, fB and fD were incubated with or without Ecb or Ecb N63E/R75EN82E (10 µg/ml) and Bb formation was analyzed by immunoblotting

SUPPLEMENTARY TABLE 1, CHAPTER 7

Strains and plasmids used in this study

Strains or plasmid	Genotype and/or description	Reference or source
RN4220	Accepts foreign DNA ($r^- m^+$)	50
Newman	Wild type *S. aureus* strain	Lab stock\
E. coli Top10F'	F´ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G λ	Invitrogen
Newman^Efb	Newman Efb::tet	This study
Newman^Ecb	Newman Ecb::tet	This study
Newman^Ecb^Efb	Newman Ecb::tet; Efb::Kan	This study
Newman^Ecb comp	Newman Ecb::tet + pCU1::Ecb	This study
Newman^Efb comp	Newman Efb::tet + pCU1::Efb	This study
Newman^Ecb^Efb comp Ecb	Newman Ecb::tet; Efb::Kan + pCU1::Ecb	This study
Newman^Ecb^Efb comp. Efb	Newman Ecb::tet; Efb::kan + pCU1::Efb	This study
Plasmids		
pAZ106	Promotorless *lacZ* insertion vector ; Em^r	51
pDG792	Kan-cassette containing vector	52
pDG1513	Tet-cassette containing vector	53
pCU1	Complementary vector for *S. aureus*	54
pCU1::Ecb	pCU1 containing the *ecb* gene plus 100 bp upstream sequence	This study
pCU::Efb	pCU1 containing the *efb* gene plus 100 bp upstream sequence	This study

SUPPLEMENTARY TABLE 2 CHAPTER 7

Oligonucleotides

Oligonucleotides	Sequence 5'-3'
EcbUPFor	CGA CGG ATC CGA AAC AAT CAG TCA TAC
EcbUPRev	ATA ACT GCG GCC GCG TGT GTT GCA ACA GTT CTT G
EcbDOWNFor	CCG GTA CCG TAA AGC ACA AAG AGC TG
EcbDOWNRev	ACA TGA ATT CTA TTT GTA ACC AAA TAG CTC
TetF	ATA ACT GCG GCC GCG GCG GAT TTT ATG ACC GAT GAA G
TetR	CCG GTA CCT TAG AAA TCC CTT TGA GAA TGT TT
EfbUPFor	CGA CGG ATC CGA CAC TCT TTA TGG GTG TGG
EfbUPRev	ATA ACT GCG GCC GCG TGG ACG TGC ACC ATA TTC G
EfbDOWNFor	CCG GTA CCG AAT ATG GTG CAC GTC C
EfbDOWNRev	ACA TGA ATT CTA GCA TCA GCC ATT GAT ACG
KanF	ATA ACT GCG GCC GCG GAA AAC CCA GGA CAA TAA CC
KanR	ACA GGT ACC CTC GGG ACC CCT ATC TAG
EcbF	CCC AAG CTT GGG TTG ATT ATT TGG TTA AAA
EbR	GGA ATT CCT ACC TTT GGA TAT AGC AA
EfbF	CCC AAG CTT GGG TGG GTC ACA ATA TTT AAC
EfbR	GGA ATT CCT AAC ATA ACA TTT AAT TA