NOVEL EVASION STRATEGIES OF *STAPHYLOCOCCUS AUREUS* AGAINST THE HUMAN INNATE IMMUNE RESPONSE

Dissertation

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SUMMARY

There is an ongoing competition between the human host and pathogenic microbes. The human immune system, especially the immediately acting innate immune response, recognizes pathogens, prevents their spreading, and eliminates them from the body. By contrast, certain pathogens have developed sophisticated strategies to evade and suppress the host immune response.

This thesis focuses on the functions of the plasma proteins apolipoprotein E (apoE) and plasmin in the interactions between the human host and the pathogen *Staphylococcus aureus.* Here, it is shown that antimicrobial peptides (AMPs) were generated from the lipid transporter apoE upon cleavage by neutrophil elastase. The antimicrobial activity was located to the heparin-binding site of the LDL-receptor-binding domain of apoE. The full-length protein had no antibacterial, but opsonic activity. Additionally, a new function of the human plasma protease plasmin, the key enzyme of fibrinolysis, is reported. The serine protease degraded the anaphylatoxin and AMP C3a and the apoE-derived AMP SHL14. Thereby plasmin terminated bactericidal activity and may facilitate clearance of effector molecules that are no longer needed after infections.

Plasminogen, the precursor of plasmin, is bound and subsequently activated by *S. aureus*, a common commensal, but also a clinically important pathogen. Here, a novel type of plasminogen-binding proteins, unique for *S. aureus*, is shown. The staphylococcal proteins Sbi and Efb recruited plasminogen together with the complement component C3. Plasminogen, fixed in these complexes, remained accessible to the human activator uPa and staphylokinase for conversion into plasmin. In the presence of Sbi or Efb, plasmin-mediated degradation of C3 and C3b was accelerated likely due to conformational changes in C3 and C3b induced by the staphylococcal proteins. Thus, *S. aureus* efficiently inactivates complement activity. Plasmin and the staphylococcal metalloprotease aureolysin were also used by *S. aureus* to degrade the AMP SHL14 and prevented cell damaging. Furthermore, both proteases proteolytically inactivated the anti-opsonic activity of apoE.

Taken together, apoE and plasmin(ogen) have different functions in the interactions between host and pathogen. ApoE is part of the immune response against *S. aureus* and thus degraded by the pathogen. By contrast, plasmin restricts immune reactions and its activity is therefore exploited by *S. aureus.*

ZUSAMMENFASSUNG

Der Mensch befindet sich in einem andauernden Wettstreit mit krankheitsauslösenden Mikroorganismen. Das humane Immunsystem, insbesondere das schnell agierende angeborene Abwehrsystem, erkennt Krankheitserreger, verhindert ihre Ausbreitung und beseitigt diese gezielt. Allerdings können bestimmte Pathogene der Immunantwort entgegenwirken und diese unterdrücken.

Diese Promotionsarbeit befasst sich mit den Funktionen der Plasmaproteine Apolipoprotein E (apoE) und Plasmin bei den Interaktionen zwischen Mensch und dem Pathogen *Staphylococcus aureus.* Es wurde gezeigt, dass antimikrobielle Peptide (AMPs) aus dem Lipid-transportierenden Protein apoE durch Verdau mit Neutrophilen-Elastase hervorgehen. Die antibakteriell wirkende Sequenz war in der Heparinbindestelle der LDL-Rezeptor-bindenden Domäne von apoE lokalisiert. Das ungeschnittene Protein hatte keine antimikrobielle Aktivität, wirkte aber als Opsonin. Weiterhin wurde eine neue Funktion für die Serinprotease Plasmin, das Hauptenzym der Fibrinolyse, beschrieben. Plasmin zerschnitt das Anaphylatoxin und AMP C3a und das von apoE-abgeleitete AMP SHL14. Dadurch inhibierte Plasmin die zellschädigende Aktivität, was vermutlich nach einer abgelaufenen Infektion von Bedeutung ist.

Plasminogen, der Vorläufer dieser Protease, wird von *S. aureus* gebunden und zu Plasmin aktiviert. *S. aureus* ist ein weitverbreiteter Kommensale, der jedoch auch eine Vielzahl von schweren Erkrankungen hervorrufen kann. In dieser Arbeit wurde ein neuer Typ von Plasminogen-Bindung, der einzigartig für *S. aureus* ist, gezeigt. Die Staphylokokkenproteine Sbi und Efb banden Plasminogen zusammen mit der Komplementkomponente C3. Plasminogen, welches in diesem Komplex fixiert war, konnte durch den humanen Aktivator uPa und durch Staphylokinase zu Plasmin umgewandelt werden. Durch die Anwesenheit von Sbi und Efb wurde der Abbau von C3 und C3b durch Plasmin verstärkt. Es ist davon auszugehen, dass dieser Effekt durch Konformationsänderungen in C3 und C3b, die durch Sbi und Efb verursacht wurden, ausgelöst wird. Folglich inaktiviert *S. aureus* in effizienter Weise das Komplementsystem. Plasmin und die Staphylokokkenprotease Aureolysin wurden auch von *S. aureus* genutzt um das AMP SHL14 abzubauen und damit die durch AMPs hervorgerufene Zellschädigungen zu verhinderte. Weiterhin degradierten beide Proteasen apoE und verhinderten damit die opsonisierende Wirkung dieses Lipoproteins.

Zusammenfassend wurde gezeigt, dass apoE und Plasmin(ogen) unterschiedliche Aufgaben bei den Interaktionen zwischen Wirt und Pathogen übernehmen. ApoE ist Teil der Immunantwort gegen *S. aureus* und wird deshalb vom Pathogen abgebaut. Plasmin hingegen kontrolliert verschiedene Immunreaktionen und seine proteolytische Aktivität wird von *S. aureus.* ausgenutzt.

ABBREVIATIONS

Ab	antibody
AMP	antimicrobial peptide
ApoE	apolipoprotein E
Au	Aureolysin
C	complement protein
C1IN	C1 inhibitor
C3aR	C3a-receptor
C5aR	C5a-receptor/CD88
CD	cluster of differentiation
CFUs	colony forming units
CHIPS	chemotaxis inhibitory protein of *S. aureus*
CR	complement receptor
CRASP	complement regulator acquiring surface protein
CRP	C- reactive protein
C-terminus	carboxy-terminus
DNA	deoxyribonucleic acid
εACA	epsilon aminocaproic acid
ECM	extracellular matrix
Efb	extracellular fibrinogen-binding protein
Efb-C	Efb carboxy-terminal fragment
e.g.	exempli gratia, for example
Ehp	Efb-homologous protein
F	coagulation factor
FH	factor H
HMWK	high molecular weight kininogen
HNE	human neutrophil elastase
HNP	human neutrophil peptide
HSA	human serum albumin
Ig	immunoglobulin
IL	interleukin
kDa	kilo Dalton
LDL	low density lipoprotein
LPS	lipopolysaccharide

LSM	laser scanning microscopy
M	molar
MASP	MBL-associated serine protease
MBL	mannan-binding lectin
MΦ	macrophage
MRSA	methicillin-resistant S*taphylococcus aureus*
NK	natural killer
N-terminus	amino-terminus
PAI	plasminogen activator inhibitor
PAMP	pathogen-associated molecular pattern
PDB	protein data bank
PL	plasmin
PLG	plasminogen
PMN	polymorphonuclear neutrophil
PRR	pattern recognition receptors
RNA	ribonucleic acid
SAK	staphylokinase
Sbi	staphylococcal binder of IgG
SCIN	staphylococcal complement inhibitor
SHL14	apoE-derived AMP
SpA	staphylococcal protein A
SPR	surface plasmon resonance
SSL	staphylococcal superantigen-like protein
TED	thioester containing domain
TCC	terminal complement complex
tPa	tissue type plasminogen activator
TNFα	tumor necrosis factor alpha
uPa	urokinase type plasminogen activator
VRSA	vancomycin-resistant *Staphylococcus aureus*

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1 INTRODUCTION

1.1 Arms race

Every day, the human immune system is attacked by a variety of microorganisms including bacteria, viruses, fungi, and parasites. Although some microbes harmlessly colonize our body and are important for supporting our immune defense (1), others take part in the ongoing competition between the human host and pathogens. This process is termed evolutionary “arms race”. Originally, the term “arms race” was defined as “competition among nations in which each tries to become militarily stronger than the other” (Oxford Advanced Learners Dictionary). But today the term is used more generally to describe the situation, when two parties with equal powers face each other, and struggle to gain advantage over the opponent.

On one hand, the human immune system fights unwanted intruders by numerous defense mechanisms including innate and adaptive immune responses. On the other hand, pathogens like *Staphylococcus aureus* have developed sophisticated strategies to successfully evade those defense mechanisms (2). In addition to the human immune response, antibiotics are used to kill pathogenic bacteria. But again, some bacteria have evolved resistance. Certain *S. aureus* strains have acquired multiple-drug resistance (3) illustrating the importance of research on this bacterium. This study focuses on the innate immune response and the evasion mechanisms used by *S. aureus.*

1.2 The immune system versus pathogens

The immune system is based on several effector mechanisms, such as physical barriers including epidermis, respiratory epithelium, vascular endothelium, and mucosal surfaces. Whenever these barriers are breached, e.g. due to an injury, host innate and adaptive immune defenses target foreign intruders (4). Adaptive immunity is mediated by T- and B-cells, which present a unique antigen receptor out of a large and diverse repertoire on their surface. However, activation, differentiation, and amplification of cells carrying a specific receptor (clonal expansion) takes three to five days (5). During this time, the host is protected by the actions of the fast acting innate immunity, which uses a defined repertoire of germline-encoded antigen receptors. These receptors recognize a limited number of evolutionarily conserved microbial structures termed pathogen associated molecular patterns (PAMPs) that are essential for the survival of the microorganisms. PAMPs are invariant and not associated with mammalian cells, such as LPS, peptidoglycan, and sugar mannose (4). PAMP-recognition receptors (PRRs)

are found on a variety of human cells, including macrophages, dendritic cells, and B-cells, but also soluble pattern recognition receptors circulate in the blood, such as complement proteins that initiate the complement cascade or act as opsonins. Key players of the innate immune response are cationic antimicrobial peptides (AMPs), phagocytes, and complement proteins (5).

1.2.1 Antimicrobial peptides

Peptides that exert cytotoxic activity against diverse microorganisms including bacteria, fungi, some viruses, and eukaryotic parasites are found in plants, animals, and some bacteria. In humans, AMPs are synthesized by cells within the epithelial barriers or circulating white blood cells (6, 7). Additionally, AMPs can be generated by proteolysis of plasma proteins. During infections, neutrophils are recruited to the site of infection and they subsequently release proteases, such as elastase (HNE) (8), which cleaves e.g. β_2-glycoprotein I or high molecular weight kininogen (HMWK) into AMPs (9, 10). Interestingly, structural motifs associated with heparin affinity confer antimicrobial activity. Thus, synthetic peptides derived from the heparin-binding motifs XBBBXXBX or XBBXBX (X represents hydrophobic and B represents basic amino acids; proposed by Cardin and Weintraub) act antibacterial (11).

AMPs mostly have a positive net-charge caused by lysine and arginine residues and vary in size, structure, and mode of action. These peptides contain 12 to 50 amino acids (12) and are, based on their structure, categorized into four classes: (i) linear α-helical peptides, (ii) peptides that contain disulfide-linked β-sheets, (iii) β-hairpins or loops, and (iv) extended peptides. The most abundant AMPs contain an α-helical structure, such as LL-37, or β-sheets like defensins (FIG. 1) (13, 14). LL-37 consists of 37 amino acids and is derived from the 18 kDa precursor CAP18, which is secreted by several cells including neutrophils, monocytes, NK-cells, and epithelial cells (15). Defensins are 3.5-6 kDa peptides that are characterized by three disulfide bridges between six conserved cysteine residues e.g. the α-defensin HNP-1 (human neutrophil peptide 1) (16).

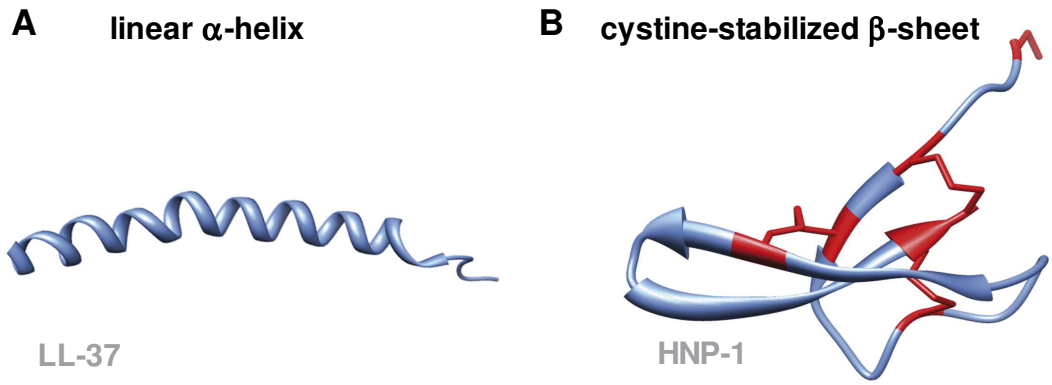

FIG. 1: Secondary structures of LL-37 and HNP-1.
Three-dimensional models of the secondary structures of the α-helical peptide LL-37 (A) and the α-defensin HNP-1. (B) The backbones of the molecules are shown in blue and disulfide bridges are shown in red. These images were prepared from Protein Data Bank (PDB) files 2K6O (17) and 3HJ2 (Wei *et al.* to be published) using Chimera software.

AMPs target microorganisms by their negative net charge caused by anionic components, such as peptidoglycan, teichoic acids, lipid A, and phospholipids. Upon membrane interaction, AMPs often form amphipathic structures and induce killing by perturbation of the cytoplasmic membrane bilayer or by acting on anionic intracellular targets, such as DNA or RNA (12, 18-20). The bactericidal activity of AMPs can be affected by physiological salt concentrations and the presence of plasma proteins (21, 22). However, mice lacking the analogue of LL-37 displayed a higher susceptibility to necrotic skin infections caused by *Streptococcus* (15). Furthermore, several AMPs were identified to exert additional immunomodulatory functions including chemotaxis of leukocytes, induction of histamine release from mast cells, and secretion of cytokines and chemokines. Besides these proinflammatory functions, some AMPs also possess immune suppressive activity by neutralizing LPS and other bacterial products (12).
AMPs can act against multidrug-resistance microorganisms, and are therefore considered as new antibiotics. AMPs possess several positive features like selective toxicity to the pathogenic target, broad spectrum of activity, neutralization of endotoxins, and the unaffectedness by classical antibiotic resistance. However, AMP therapeutics have certain disadvantages, such as the high production costs, susceptibility to proteolytic degradation, and the very high doses necessary for effective killing of microorganisms (14).

1.2.2 Phagocytes

Macrophages, dendritic cells, and polymorphonuclear neutrophils (PMNs) are professional phagocytes, which recognize and eliminate infectious agents and remove

apoptotic cells. Phagocytes detect their targets directly or indirectly by opsonins using PRRs, such as Toll-like receptors, scavenger receptors or opsonin receptors. Classical opsonins are components of the complement system like C1q, mannose-binding lectin (MBL), C3b, iC3b, and immunoglobulins (IgGs). In addition, serum proteins, such as CRP, β2-glycoprotein I, protein S, fibronectin, and vitronectin are bridging molecules, which bind to apoptotic cells and phagocyte receptors to facilitate phagocytosis (23, 24). Upon binding, the targets are internalized and trapped in a phagosome that fuses with a lysosome to form the phagolysosome. The lysosome contains proteolytic enzymes like elastase or lysozyme, cationic antimicrobial peptides and proteins, reactive oxygen species, several lipases, nucleases, glycosidases, and phosphatases, which lead to the destruction of large complex structures, such as microbial intruders (25). Among all phagocytes, PMNs are the best equipped and the first to arrive at the site of infections (26).

1.2.3 The complement system

The complement system was originally discovered as the heat-labile part of immune reactions that “complement” the killing of bacteria in serum. This immune surveillance system comprises over 30 plasma and cell-bound proteins. Complement is the first line of defense against microbial intruders. It also links innate and adaptive immunity by helping T- and B-cells to eliminate pathogens and facilitates the clearance of apoptotic cells, as well as immune complexes, from the circulation (27-30).

The alternative pathway is constantly activated by spontaneous hydrolysis (tick over) of C3 to C3(H_2O), which recruits factor B. Bound factor B is cleaved by the plasma protease factor D into Ba and Bb forming the fluid-phase C3 convertase C3(H_2O)Bb. In addition, the classical pathway is activated by the recognition of immune complexes on target cells by C1q. Thereby the C1q associated serine proteases C1r and C1s are activated. The lectin pathway is initiated when MBLs or ficolins recognize carbohydrate moieties leading to auto-activation of MBL-associated serine proteases (MASPs). The activated serine proteases of the classical and lectin pathways proteolytically activate C4 to C4a and C4b. The cleavage product C4b covalently attaches to the target surface, binds C2, which is also cleaved, and assembles the C3 convertase (C2a4b). All three complement activation pathways merge in the formation of C3 convertases, which cleave the central component C3 into C3a and the opsonin C3b (29-31). C3a has several physiological functions such as antimicrobial activity against Gram-positive and –negative bacteria. The molecule shares features of α-helical AMPs and defensins. C3a

is a cationic peptide of 77 amino acid residues containing four α-helical structures linked by three disulfide-bridges (FIG. 2). Additionally, C3a acts as potent chemoattractant for phagocytes, causes histamine release, and induces oxidative burst from neutrophils (30, 32, 33).

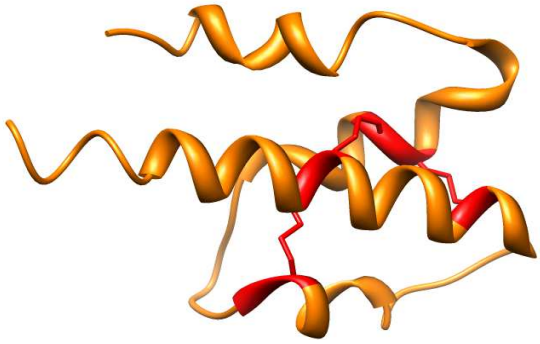

FIG. 2: Secondary structure of C3a.
Three-dimensional model of the C3a secondary structure based on PDB file 2A73 (34). The backbone is shown in orange and disulfide bridges are colored in red.

C3b covalently attaches to the target cell surface, amplifies complement activation by forming the solid-phase C3 convertase (C3bBb), and labels the cells to support phagocytosis (opsonization). Furthermore, C3b deposition leads to formation of C5 convertases, which cleave C5 into C5a and C5b. C5a is a potent anaphylatoxin, which attracts phagocytes to infection sites and is also involved in activation of phagocytes, generation of oxidants, and release of granule-based enzymes (35). C5b associates with C6, C7, C8, and C9 and forms the terminal complement complex (TCC) on the target cell surface leading to lysis of certain cells, such as Gram-negative bacteria (FIG. 3) (27-29).

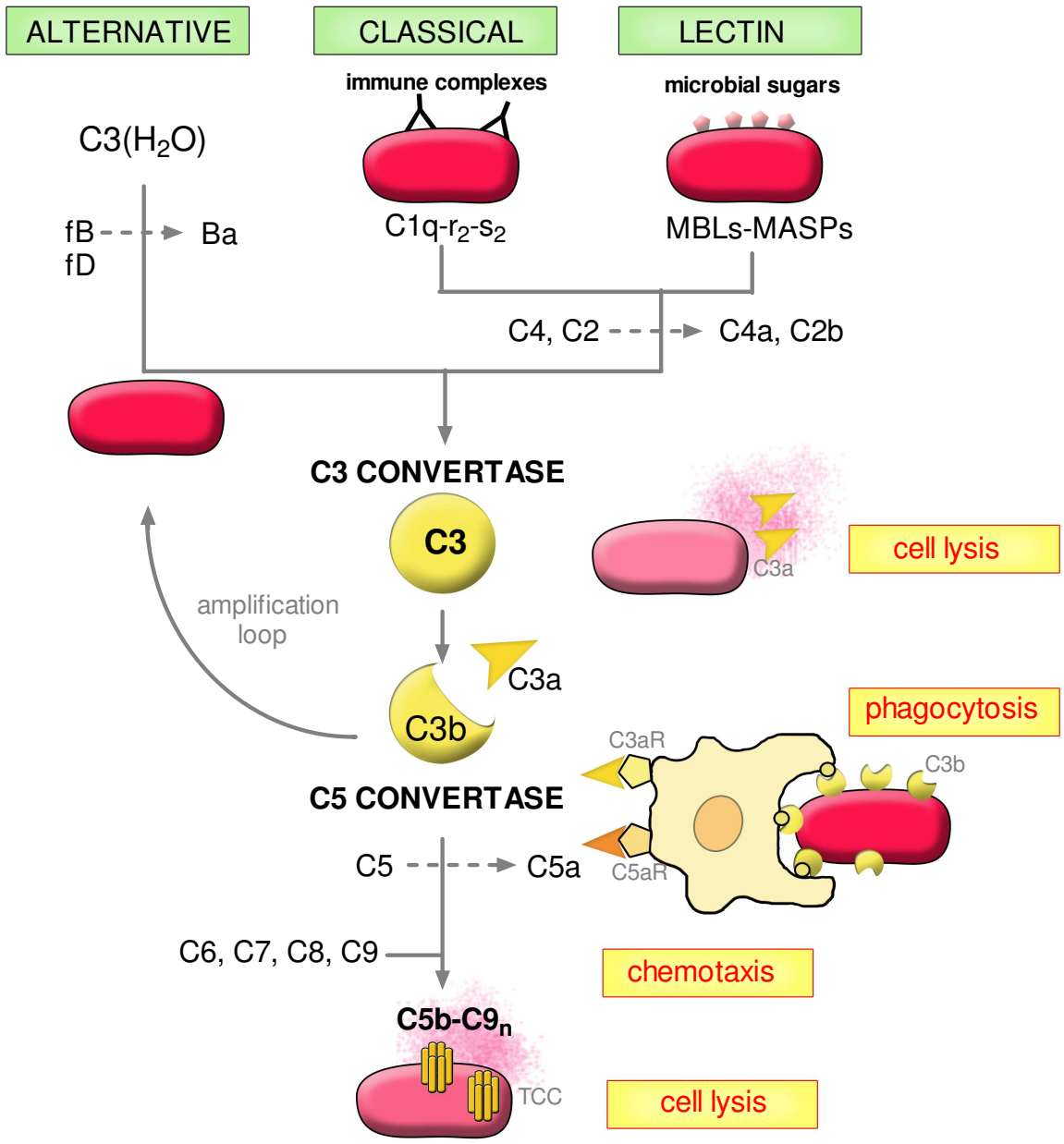

FIG. 3: Scheme of the human complement system.
The complement system is initiated by three different pathways. The alternative pathway is continuously activated by spontaneous hydrolysis of C3, the lectin pathway is activated by binding of mannan-binding lectin (MBL) to mannose residues, and the classical pathway is initiated by binding of the C1-complex to antigen bound antibodies. All pathways converge in the cleavage of C3 by C3-convertases. The cleavage product C3a exerts antimicrobial activity. C3b deposits on targets cells, marking them for phagocytosis, and forming C5-convertases, which cleave C5 into C5a and C5b. C5a and C3a are small chemoattractant molecules that recruit phagocytes to the site of infection. C5b together with C6, C7, C8 and multiple C9 molecules forms a lytic terminal complement complex (TCC). Schematic illustration is modified after (29) and (31).

To protect host cells from the deleterious effects of complement, the cascade is tightly regulated. Initiation, amplification, and effector functions are supervised by soluble and membrane-bound regulators. The amplification of C3 convertases and the subsequent

cleavage of C3 are especially tightly regulated. C3 is a 187 kDa protein that consists of an α- and β-chain linked by two disulfide bridges. The molecule is arranged in 8 macroglobulin domains, a linker domain, an anaphylatoxin domain, a CUB (complement C1r/C1s, Uegf, Bmp1) domain, a thioester containing domain (TED), and the C345c domain. In the intact protein the reactive thioester group is buried in the molecule. Upon complement activation C3 and its fragments undergo various conformational changes (FIG. 4). C3 convertases release C3a, the anaphylatoxin domain (shown in orange), from the molecule. The remaining 12 domains, especially the CUB domain (shown in cyan) and TED (shown in green), rearrange and the thioester moiety covalently attaches to the target surface or reacts with water. In contrast to the rather inert C3, C3b interacts with factor B to initiate the amplification loop of the alternative pathway or binds complement regulatory proteins, such as the main fluid phase regulator of the alternative pathway factor H (31). Factor H is a 150 kDa glycoprotein that is continuously produced by liver cells and circulates with a concentration between 500-800 µg/ml in human plasma. This protein has three complement regulatory functions, factor H (i) inhibits the alternative convertase assembly, (ii) accelerates the decay of the C3 convertase, and (iii) serves factor I as cofactor for the proteolytic inactivation of C3b yielding iC3b (36). iC3b is recognized by complement receptors 3 and 4 (CR3/CD11b-CD18 and CR4/CD11c-CD18), or further degraded by factor I and the membrane-bound cofactor CR1 (CD35) into C3c, and C3dg (31, 37). C3dg remains covalently linked to the target surface and is recognized by CR2 (CD21) expressed on B-cells, follicular dendritic cells, and immature T-cells. Antigen-bound C3dg lowers the threshold for B-cell activation by the antigen receptor and thereby accelerates adaptive immunity (38).

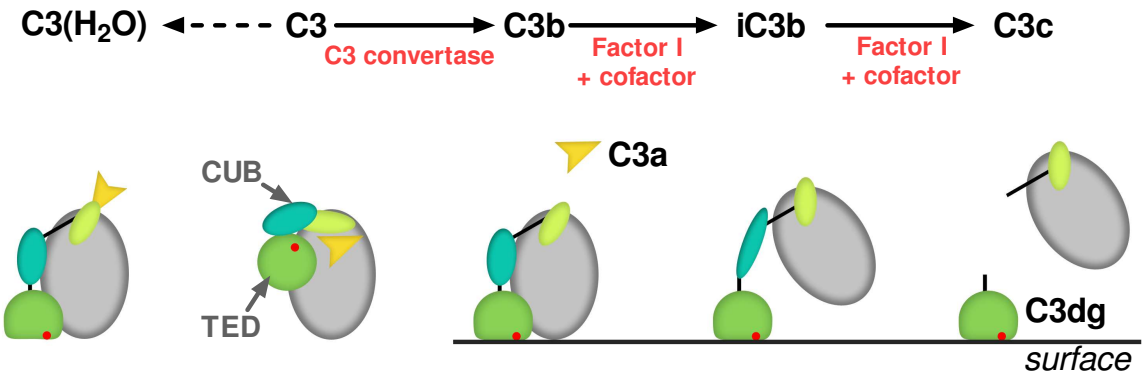

FIG. 4: Schematic presentation of the structures of C3 and its fragments.
Spontaneous hydrolysis of C3 leads to C3(H_2O). C3 is cleaved by the C3 convertase into C3a and C3b, which attaches to a target surface. Processing by factor I and an appropriate cofactor generates iC3b and C3f (not shown), and then C3c and C3dg. This figure is adopted from (31) with minor modifications.

1.2.4 The coagulation system

Besides the classical components of innate immunity, coagulation is believed to be also involved in the host response to bacterial infections (39, 40). Coagulation is a cascade of plasma proteases that forms blood clots at the site of injury to stop extensive bleeding. Additionally, these clots trap bacteria and impair their spreading (FIG. 5).

Blood coagulation is initiated via an intrinsic pathway (all components are present in blood) or by an extrinsic pathway (one component is required in addition to the circulating components). The extrinsic pathway is activated when tissue factor, normally hidden in the subendothelial layer, is exposed to blood due to tissue damage. Tissue factor nonproteolytically activates FVII to FVIIa and, complexed with FVIIa, converts FX and FIX to the active forms FXa and FIXa, respectively. The intrinsic activation pathway is initiated by contact to an anionic or hydrophilic solid surface, which converts the zymogen FXII (Hageman factor) into the active form FXIIa in a kallikrein-HMWK-dependent fashion. Sequentially, the coagulation factors FXI, FIX, and FVIII are activated and a tenase complex is formed. Extrinsic and intrinsic pathways merge into the activation of FX to FXa, which forms a prothombinase complex with FVa leading to the proteolytic activation and conversion of prothrombin to thrombin. In the final step, thrombin hydrolyzes fibrinogen to fibrin, which oligomerizes and causes plasma coagulation. Activated FIXa together with FVIIIa amplifies the clotting reaction through acceleration of FX activation (39, 41).

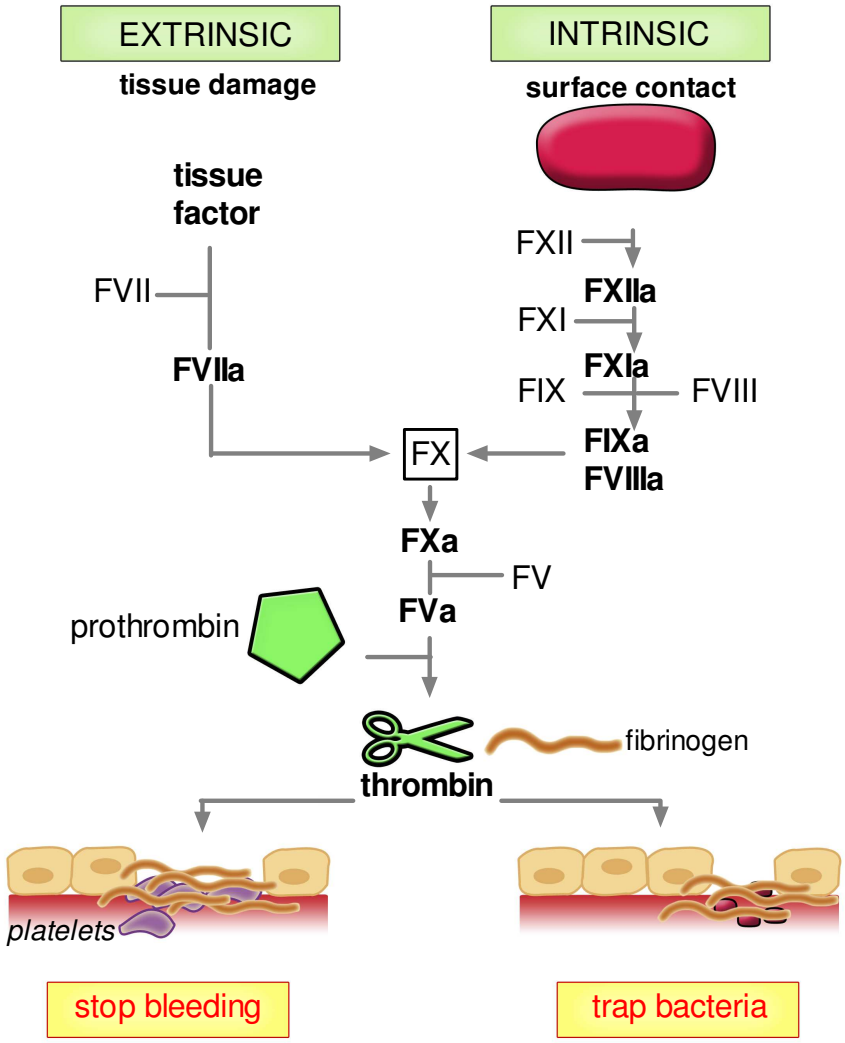

FIG. 5: Scheme of the human coagulation system.
Blood coagulation can be initiated by tissue damage (extrinsic pathway) or anionic surfaces (intrinsic pathway). Activation culminates in generation of thrombin. Thrombin cleaves fibrinogen yielding cross-linked fibrin clots, which stop bleeding and trap pathogens. Scheme is modified after (39) and (40).

To prevent uncontrolled bleeding or thrombosis, pro- and anticoagulant factors need to be balanced. Coagulation is negatively regulated by the anticoagulation system including regulators at each level which prevent or slow the propagation of fibrin clots. In addition, the fibrinolytic system, also known as plasminogen-plasmin system, plays a key role in maintaining the equilibrium between coagulation and fibrinolysis (39, 42).

1.2.5 The plasminogen-plasmin system

The primary function of the fibrinolytic system is the dissolution of existing fibrin clots to allow wound healing processes. Additionally, plasmin, the main fibrinolytic enzyme, degrades components of the extracellular matrix (ECM) to facilitate eukaryotic cell

migration, and proteolytically activates prohormones, growth factors and metalloproteases (39, 43, 44).

The human plasma protein plasminogen is the zymogen of the serine protease plasmin. Plasminogen is primarily synthesized by liver cells (45), and circulates in plasma with a concentration of ~2 µM (46). Plasminogen, whose three-dimensional structure was recently solved by Law *et al.* (47), has a molecular weight of 92 kDa and consists of five kringle domains and a protease domain (

FIG. 6) (48). Each of the kringle domains, which are named after the Danish pastry, consists of 78-80 conserved residues that form a triple-loop structure linked by disulfide bridges. The kringle domains contain the binding site for lysine residues in fibrin, components of the ECM, and several cell receptors. The proteolytic domain is composed of 230 residues containing the catalytic triad His_{603}, Asp_{646}, and Ser_{741}

Conversion of plasminogen to plasmin is facilitated by human or bacterial activators (43). The two human activators uPa (urokinase-type) and tPa (tissue-type) are serine proteases that cleave the Arg_{561}-Val_{565} bond in plasminogen. The resulting plasmin consists of two disulfide-linked polypeptide chains (49). Plasmin preferably cleaves peptide bonds next to lysine or arginine residues (50) and has a broad-substrate specificity including fibrinogen, fibrin, components of the ECM (laminin, vitronectin, fibronectin), and complement components, such as C1, C2, C3, C3b, C4, and C5 (51-55).

Plasminogen occurs in various modifications. The native form Glu-plasminogen contains an N-terminal 8 kDa preactivation peptide that is released by plasmin resulting in Lys-plasminogen. Lys-plasminogen binds with higher affinity to target molecules and receptors and also facilitates conversion to plasmin (56). Additionally, limited proteolysis of plasminogen yields mini-plasminogen comprising kringle 5 and the protease domain. Plasminogen is also subject to posttranslational modification that consists of Asn_{289}-, Thr_{346}-, Ser_{248}-, Thr_{339}-linked glycosylation, and O-linked phosphorylation (44). The plasminogen-plasmin system is tightly regulated by inhibition of plasminogen activators (plasminogen activator inhibitor (PAI)-1 and -2) or by inhibition of the activated protease plasmin (α_2-antiplasmin and α_2-macroglobulin) (57).

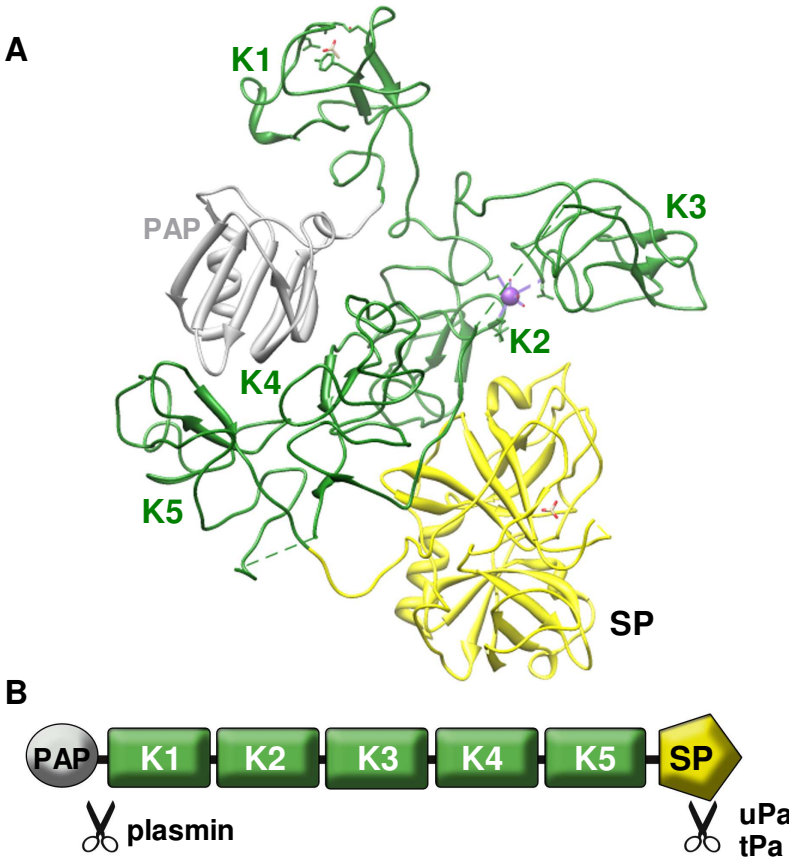

FIG. 6: Structure of plasminogen.
(A) Three-dimensional structure of plasminogen prepared from PDB file 4DUR (47). The pre-activation peptide is shown in grey, kringle domains are green, and the serine protease domain is yellow. (B) The schematic structure of plasminogen is modified after (44). Cleavage sites for plasmin, uPa, and tPa are indicated by scissors.

1.2.6 Cross-talk between complement, coagulation, and fibrinolysis

Complement, coagulation, and its antagonist, the fibrinolytic system, have several crossover points linking the three protein networks together (40, 52, 58, 59). In particular, the classical complement activation pathway is also initiated by clotting factor FXIIa, which activates the C1-complex (60). Complement C5a induces tissue factor activity and may therefore be involved in the initiation of the extrinsic coagulation pathway (61). Additionally, serine proteases of the coagulation and fibrinolytic system were reported to cleave components of the complement cascade e.g. thrombin, kallikrein, FXa, FXIa, FIXa, and plasmin nontraditionally activate complement by cleaving C3 and C5 resulting in C3a and C5a generation (52). Moreover, C1 inhibitor (C1IN) regulates both, the complement and coagulation cascades, by inhibiting C1r, C1s, MASP2, plasma kallikrein, and FXIIa (62).

1.2.7 Apolipoprotein E

Apolipoprotein E (apoE) is a 34 kDa glycoprotein expressed by many tissues including liver, brain, skin and tissue macrophages. This protein is associated with a variety of diseases including the Alzheimer's disease, dementia, diabetes, kidney- and cardiovascular disorders. ApoE is composed of two distinct functional domains that are

linked by a region of random like structure. The N-terminal domain forms a four-helix bundle, in which helix 4 contains the binding site for heparin and for receptors of the LDL-receptor family. The C-terminal domain consists of amphipathic α-helices that constitute the major lipid-binding site and an additional heparin-binding site. ApoE is polymorphic and exhibits three isoforms, apoE2, the most abundant E3, and E4, which differ at the two residues 112 and 158 (E3: cys_{112}, arg_{158}, E4: arg_{112}, arg_{158}, E2: cys_{112}, cys_{158}) (FIG. 7). Although there is only a single amino acid substitution, the structure and thus the function of apoE2 and E4 are dramatically changed. ApoE2 binds less to the LDL-receptor and apoE4 preferentially binds VLDLs instead of HDLs. Both dysfunctions are associated with diseases, apoE2 with the genetic disorder type III hyperlipoproteinemia and atherosclerosis and apoE4 with atherosclerosis and the Alzheimer's disease (63, 64).

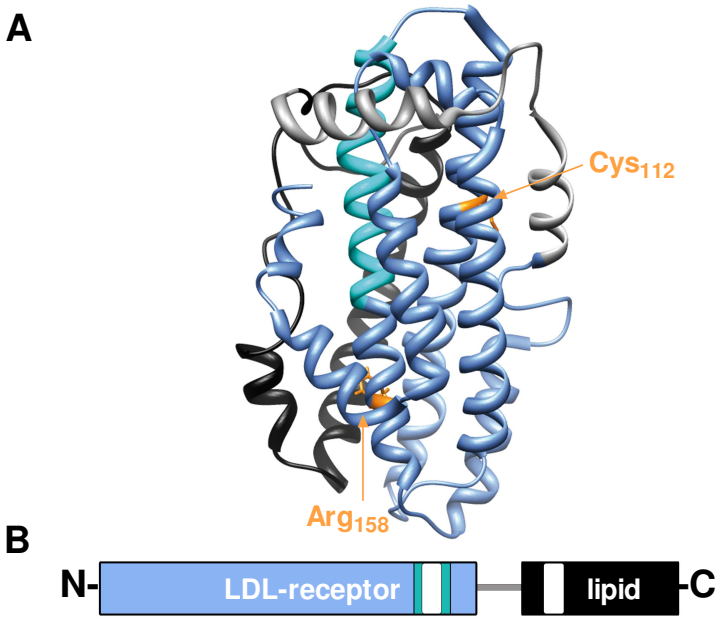

FIG. 7: Structure of apoE3.
(A) Three-dimensional structure of apoE prepared from PDB file 2L7B (65). The LDL-receptor domain is shown in blue (LDL-receptor-binding site in aquamarine) and the lipid-binding domain in black. Residue 112 and 158 are shown in orange. (B) Schematic structure of apoE. Heparin-binding motifs are white.

ApoE was originally described as a component of triglyceride-rich lipoproteins. The protein transports and delivers triglycerides and cholesterol to extrahepatic cells or the liver (66). Over the last 30 years further functions are discussed. Mice lacking apoE display susceptibility to bacterial infections indicating that apoE contributes to immunity (67-69). ApoE was found to modulate NO, IL-12, TNF-α production, MHCs expression (70), lipid antigen presentation by CD1d (71), uptake of apoptotic cells (72) and β-amyloid (73) by macrophages. Additionally, synthetic peptides derived from the LDL-receptor-binding site showed antibacterial (74) and antiviral activity (75, 76). However, their natural occurrence remained unclear. Recently, apoE was detected on the surface of *S. aureus* (77).

1.3 Pathogens versus the innate immune response

1.3.1 *Staphylococcus aureus*

S. aureus is a Gram-positive coccus-shaped bacterium that lives as a commensal on the skin, nose and throat. Approximately 30% of humans are asymptomatically colonized by this bacterium. However*, S. aureus* also causes diverse complications including minor skin infections, endocarditis, and sepsis. Furthermore, *S. aureus* is a major cause of food poising caused by the heat resistant enterotoxin A (78). The bacterium belongs to the leading cause of nosocomial infections and rapidly develops antibiotic resistance. The most severe infections are caused by the increasing number of MRSA strains, which are resistant to the entire class of β-lactam antibiotics including methicillin (79). These staphylococcal infections are treated with vancomycin, an antibiotic that disrupts the synthesis of the cell wall component peptidoglycan. However, some *S. aureus* strains (VRSA) have already been reported to be resistant (3). In order to catch up with the bacteria in the evolutionary "arms race" and develop new antibacterial substances, the staphylococcal pathogenicity needs to be understood.

1.3.2 Staphylococcal evasion strategies – an overview

To establish in the human host, *S. aureus* circumvents the immune defenses, especially the immediately acting innate immunity. The bacterium targets all branches of innate immune response using an arsenal of virulence factors including (i) small nonproteolytic molecules that directly inhibit host effector proteins, (ii) proteases and activators for host proteases, and (iii) surface-bound molecules that acquire and exploit host regulators (2, 26, 80).

S. aureus counteracts **AMPs** by at least four mechanisms: (i) alteration of membrane structure and fluidity e.g. by increase of longer chain unsaturated fatty acids, (ii) reduction of the negative surface net charge by modification of teichoic acids with D-alanine or phospholipids with L-lysine, (iii) secretion of trapping molecules like staphylokinase (SAK), which binds and inhibits α-defensins, and (iv) proteolytic degradation of AMPs by secreted proteases (

FIG. 8) (7, 19).

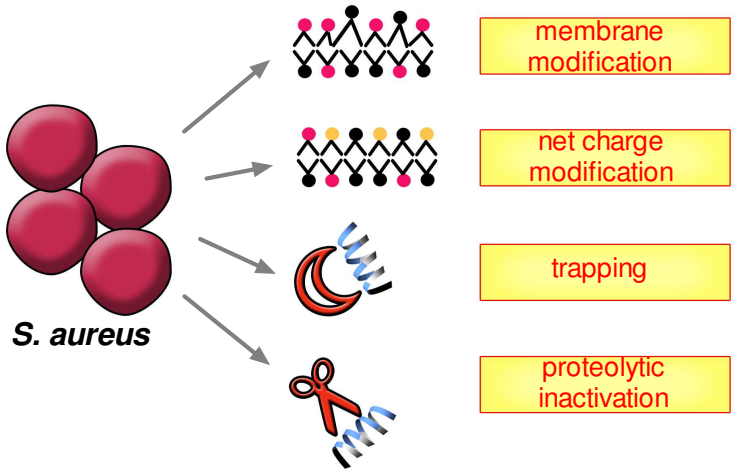

FIG. 8: Staphylococcal evasion strategies against AMPs.
S. aureus counteracts AMPs by several mechanisms. The pathogen modifies its membrane structure or net charge and secretes trapping molecules or proteases that inactivate AMPs (19).

Moreover, *S. aureus* thwarts **phagocytes** in multiple ways. *S. aureus* secretes CHIPS (chemotaxis inhibitory protein of staphylococci), which blocks neutrophil receptors for the major chemoattractants C5a and fMLP (N-formyl-methionine-leucine-phenylalanin) (81). To evade opsonization, *S. aureus* expresses IgG-binding proteins, such as staphylococcal protein A (SpA), superantigen-like protein-10 (SSL-10), and staphylococcal binder of IgG (Sbi), which all sequester the Fc-region of IgGs (82-85). Most clinical isolates express a capsule, which reduces the uptake by neutrophils *in vitro* (86). Additionally, *S. aureus* has the ability to survive in phagocytes, e.g. by scavenging oxygen free radicals through its yellow pigment (87). By blocking the complement cascade, the bacterium inhibits chemotaxis caused by the anaphylatoxins C3a and C5a, as well as opsonization by the complement-derived opsonins C3b and iC3b.

S. aureus is a master at evading the **complement system** as the pathogen expresses specific small proteins and proteases to influence nearly every step of the cascade (FIG. 9). Initiation of the classical pathway is inhibited by IgG-binding proteins (26, 85, 88) or degradation of IgGs by SAK-activated plasmin (1.3.5). *S. aureus* interferes with complement at the C3 level by producing (i) C3-binding proteins (reviewed in chapter 1.3.3), (ii) the C3 convertase stabilizer SCIN (staphylococcal complement inhibitor) (89), and (iii) aureolysin (Au), which degrades C3 (1.3.4). The bacterium also (iv) activates the C3-degrading host protease plasmin by SAK (1.3.5), and (v) exploits the activity of the host complement regulators factor I and factor H (90-92). Moreover, staphylococcal superantigen-like protein-7 (SSL-7) binds to C5 and thereby inhibits TCC formation (93).

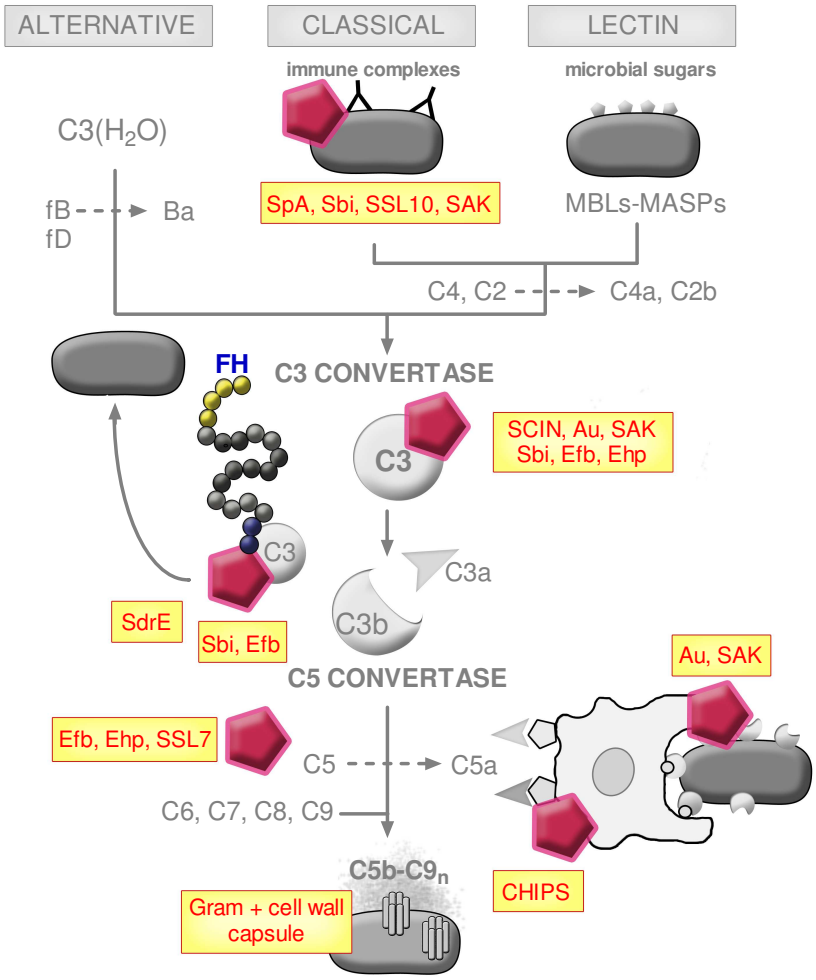

FIG. 9: Complement evasion by staphylococcal inhibitors.
S. aureus secretes a variety of small complement inhibitory molecules (shown as pink pentagons) that target certain steps of the cascade or bind the complement regulatory protein factor H (FH).

1.3.3 The C3-binding proteins Sbi and Efb

To date, three structurally related C3-binding proteins secreted by *S. aureus* have been identified (94): Sbi, extracellular fibrinogen-binding protein (Efb), and its homolog Ehp. Sbi is a ~50 kDa protein that comprises four structural domains, a proline repeat-containing linker (P) and tyrosine-rich region (Y) (FIG. 10). The protein is detected in the extracellular fractions and on the bacterial surface. Sbi was originally identified as the second IgG-binding protein of *S. aureus* and the binding site was mapped to the two N-terminal domains Sbi 1 and 2 (I-II). Pull-down experiments further showed that domains 3 and 4 (III-IV) bind complement C3d in native C3, iC3b, and C3dg and that these domains inhibited all complement pathways (90, 95, 96). Burman *et al.* proposed that Sbi 3-4 causes fluid phase consumption of C3 (95). Additionally, Sbi was found to form

a tripartite complex with C3 (C3b, C3d) and the complement regulator factor H, to exploit factor H cofactor activity for factor I-mediated inactivation of C3b (90).

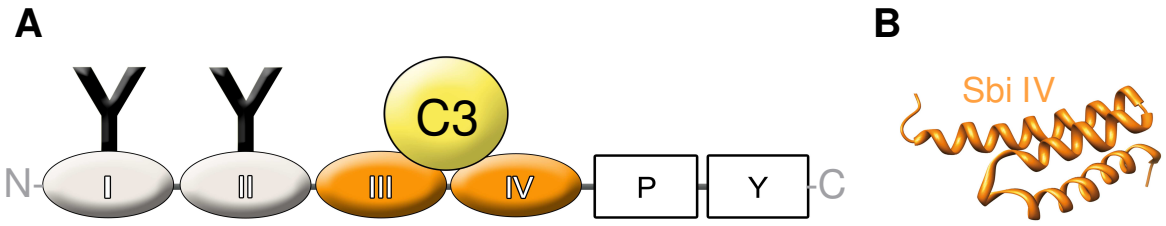

FIG. 10: Structure of Sbi.
(A) Sbi consists of four globular domains (I-IV), a proline-rich (P) and tyrosine-rich (Y) region. The N-terminal domains bind IgGs and Sbi 3 and 4 (III-IV) interact with C3. This illustration is derived from (95) and (90). (B) The three-dimensional structure of domain 4 displays an α-helical bundle. This image is prepared from PDB file 2JVG (94).

The 19 kDa Efb consists of an N-terminal fibrinogen-binding domain and a C-terminal C3-binding domain. The protein is secreted and also present on the bacterial surface (97, 98). In addition to Efb, Ehp, a ~10 kDa homologue protein (44% identity), is secreted by *S. aureus*. Efb and Ehp, like Sbi, bind various forms of C3 containing the C3d fragment (98-100). Furthermore, Efb induces conformational changes in C3 and C3b that allosterically inhibit the participation of C3 and C3b in downstream activation processes of the complement cascade (100-102). Efb-C, Ehp, and Sbi 4 mainly bind to the same residues in C3d (100, 103) and contain a three helix domain (shown in orange FIG. 10 and FIG. 11) that is also present in other staphylococcal complement inhibitors, such as SCIN (94). Efb, Ehp, and Sbi block the interaction of C3d with CR2 to inhibit a crucial link between the innate and adaptive immunity (104, 105).

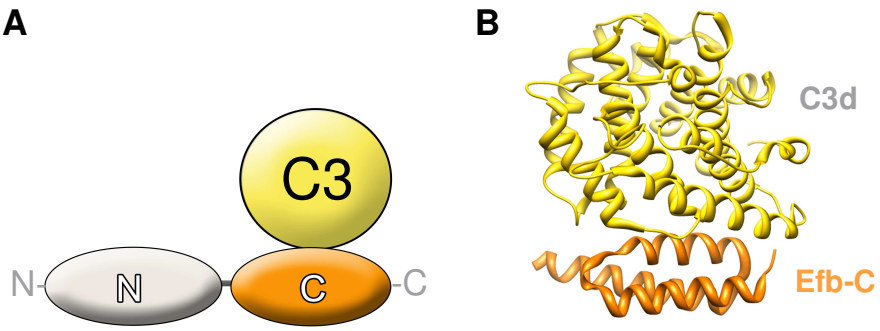

FIG. 11: Structure of Efb.
(A) Efb consists of an N-terminal fibrinogen-binding domain and a C-terminal C3-binding domain. (B). Three-dimensional structure of Efb-C in complex with C3d. Efb-C, like Sbi 4, consists of three α-helices. Figure was adapted from PDB file 3D5S (106).

1.3.4 Staphylococcal proteases

Pathogenic proteases are believed to play an important role during infections (1). The staphylococcal arsenal comprises serine (e.g. V8 protease), cysteine (staphopains) and metalloproteases (aureolysin). The V8 protease has narrow substrate specificity; however the protease inactivates the plasma protease inhibitor α_1-proteinase inhibitor, which protects tissues from enzymes of inflammatory cells, such as HNE. Additionally, V8 protease, like staphopain A/B, targets the intrinsic coagulation pathway by releasing kinins, which facilitate spreading of microorganisms and may promote influx of plasma containing nutrients (1, 107, 108).

Aureolysin is an EDTA-sensitive metalloprotease, which is secreted throughout the bacterial growth cycle to target bacterial and host proteins (109). The protease activates V8 (110) and releases a variety of surface-bound staphylococcal proteins, such as SpA, to facilitate the transition from adherent to invasive phenotype. Among the host substrates are the α-helical AMP LL-37, protease inhibitors, and components of the fibrinolytic and complement system (107, 111). For example, aureolysin activates the precursor of the human plasminogen activator pro-uPa to uPa, inactivates the plasmin inhibitor α_2-antiplasmin (112), and cleaves C3 leading to inhibition of complement-mediated phagocytosis and neutrophil activation (113).

1.3.5 Hijacking plasmin(ogen)

S. aureus, like numerous other bacteria, acquires the host regulatory protein plasminogen (2, 26, 43, 114) and uses activated plasmin to create uncontrolled proteolysis that facilitates bacterial migration across tissue barriers and accommodates nutritional demands (115). In addition, *S. aureus* benefits of plasmin-mediated degradation of the opsonins C3b and IgG (116).

Previously, three staphylococcal plasminogen-binding proteins have been identified: inosine 5′-monophosphate dehydrogenase, α-enolase, and ribonucleotide reductase (117). Remarkably, *S. aureus* activates plasminogen by several strategies: (i) the bacterium enhances the production of uPa in mammalian cells (57), (ii) secretes the proteases aureolysin, which activates the host plasminogen activator uPa (112), and (iii) produces the nonproteolytic plasminogen activator staphylokinase (SAK) (118, 119).

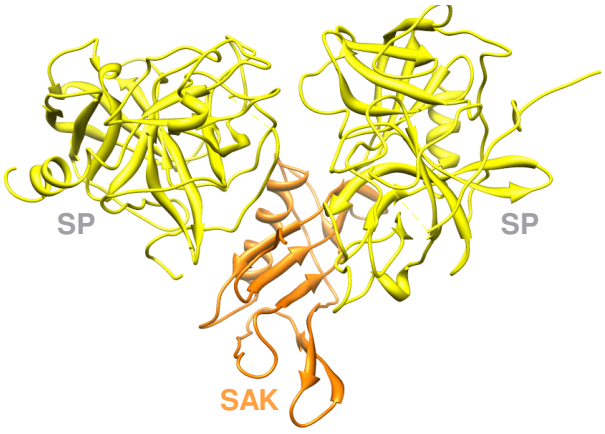

FIG. 12: Structure of SAK in complex with SP (plasminogen).
SAK, shown in orange, forms a complex with the serine protease domain (SP) of plasminogen, two SPs are shown in yellow, and non-proteolytically generates plasmin. Image is adapted from PDB file 1BUI (120).

SAK is a ~16 kDa single chain protein whose three-dimensional crystal structure consists of a central α-helix, five β-sheets, and a connecting loop (121). SAK is synthesized during the late exponential growth phase by 67% of *S. aureus* strains and detected in the supernatant as well as on the bacterial surface (122). The protein forms a complex with the serine protease domain of plasminogen leading to a conformational change in plasminogen that facilitates the activation by spontaneously formed plasmin. SAK binds stronger to substrate-bound plasminogen, especially in complex with fibrin, than to soluble plasminogen (121). Due to its thrombolytic and clot-cleaving activity, SAK is a potential therapeutic for treatment of arterial thromboses and myocardial infarctions (119). Furthermore, SAK was found to have an additional immune evasion function. The protein binds the α-defensins HNP-1 and -2, and inhibits their bactericidal activity (123).

1.4 Aims of the study

S. aureus is one of the major human pathogens that cause several severe diseases. The bacterium is a specialist in immune evasion and rapidly acquires resistance to antibiotics. To develop new antibacterial therapies, a deeper understanding of the human immune response as well as the evasion mechanisms involving staphylococcal virulence factors is required. Therefore, the functions of apoE and plasmin in the interactions between the human host and *S. aureus* will be investigated.

ApoE-deficient mice show an increased susceptibility to bacterial infections, but the underlying mechanisms is not completely understood. Thus, the role of apoE in innate immunity will be characterized. One aim of the study is to investigate the antimicrobial activity of apoE, as this plasma protein harbors amphipathic α-helices and heparin-binding motifs that are common features of AMPs. In addition, apoE was detected on the surface of *S. aureus* after exposure to human plasma. Therefore, the function of apoE on the bacterial surface will be assessed.

S. aureus successfully evades the innate immune response using several virulence factors including complement inhibitors and proteases. *S. aureus* traps and activates the host protease plasmin for degradation of the complement-derived opsonin C3b. Controversially, plasmin can also initiate complement by generating C3a; thus, the effect of plasmin on complement activity at the C3 level and the consequences for *S. aureus* will be investigated. In addition to the proteolytic inactivation of C3/C3b, the bacterium expresses the C3-binding proteins Sbi and Efb. However, it is not clear whether these proteins influence the proteolysis of C3/C3b, therefore the impact of Sbi and Efb on plasmin-mediated C3/C3b degradation will be assessed.

As some AMPs are sensitive to proteolytic degradation, plasmin activity against AMPs is analyzed and compared to aureolysin, a metalloprotease secreted by all *S. aureus* strains that degrades the α-helical AMP LL-37. Furthermore, the effects of plasmin and aureolysin on apoE-mediated immune reactions will be investigated.

The results are expected to deepen our knowledge about the functions of apoE and plasmin in innate immunity and immune evasion and provide insight into some of the mechanisms deployed by *S. aureus* to counteract the human innate immune responses.

2 MATERIALS & METHODS

2.1 Chemicals and Reagents

Unless specified otherwise, chemicals and reagents were purchased in the highest available quality from Sigma or Roth.

2.2 Proteins, peptides, and enzymes

Plasminogen (PLG) and urokinase type activator (uPa) were purchased from Haemochrom Diagnostica, and plasmin (PL) from Calbiochem. Complement protein 3 (C3) and its fragments: C3b, iC3b, C3c, and C3a from Comptech. Apolipoprotein E (apoE) was obtained from Acris or BioVision and HSA from Cleveland, Ohio. The synthetic peptides LGR11; SHL14; WGE23, and LRV30 were synthesized by Peptide 2.0 (USA); LL-37 by Sigma, and FITC-labeled SHL14 by Biomatik (Canada). Human neutrophil elastase (HNE) was obtained from Scipac and aureolysin from BioCentrum.

2.3 Media and supplements

All *E. coli* strains were cultivated in LB (Luria Broth) medium (10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl, pH adjusted to 7.2; autoclaved) or LB agar plates (LB medium, 15 g/l agar) at 37°C.

To select for clones containing the desired plasmid LB medium was supplemented with 50-100 µg/ml ampicillin (Invitrogen) or 100 µg/ml kanamycin (Roth).

S. aureus was grown in LB medium or on blood agar plates (Merck) at 37°C.

For viable OD assays, *E. coli* or *S. aureus* was grown in 3% TSB (tryptic soy broth) medium (Fluka).

2.4 Bacterial strains

Escherichia coli

E. coli DH5α was used for antimicrobial assays.

One Shot® TOP10 competent *E. coli* (Invitrogen) that lacks T7 RNA polymerase was used for characterization, propagation, and maintenance of the plasmid construct.

The *E. coli* expression strain BL21 Star™ (DE3) (Invitrogen) was used for protein expression.

Staphylococcus aureus

S. aureus Newman was used for antimicrobial assays, phagocytosis assays, and its DNA was used as template for cloning SAK and Sbi 1-2.

2.5 Plasmids and primers

TOPO Expression plasmids

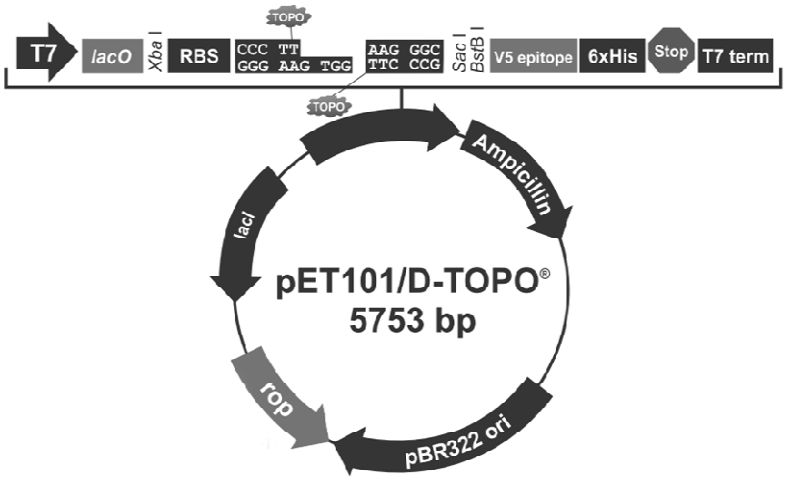

FIG. 13: Expression plasmid used for cloning of SAK and Sbi 1-2.
pET101/D-TOPO contains a GTGG overhang sequence that base pairs with the desired insert. Expressed proteins contain a C-terminal His-tag.

The expression plasmid pET101/D-TOPO® (Invitrogen) (FIG. 13) contains a C-terminal 6x His-tag coding sequence, an ampicillin resistance gene, and its expression is controlled by the T7 promoter region.

The expression plasmid pET200® (Invitrogen) contains an N-terminal 6xHis-tag coding sequence, an ampicillin as well as a kanamycin resistance gene, and its expression is controlled by the T7 promoter region. pET200 containing *sbi 1-4*, *sbi 3-4*, *efb*, or *efb-C* was previously constructed in this laboratory (M. Reuter, S. Böhm).

Primer

Sak and the *sbi 1-2* constructs were amplified using the following oligonucleotide primers (Invitrogen):

SAK	Fwd: **CACC***ATG*CTCAAAAGAGGTTTATTATTTTTAAC
	Rev: TTTCTTTTCTATAATAACCTTTGTAATTAAGTTG
Sbi1-2	Fwd: **CACC***ATG*ACAACTCAAAACAACTACGTAAC
	Rev: ATTTTGACGTTCTTTAGCTTTAGAAGATTGTACTG

The forward primers contained CACC at the 5´end to allow directional cloning and ATG as start codon. The reverse primer did not include the stop codon to allow expression of the C-terminal His-tag.

2.6 Molecular biology

Cloning strategy

Cloning of *sak* and *sbi 1-2* into pET101/D-TOPO® were performed according to manufacturer´s instructions (Invitrogen). Crucial steps are shown in FIG. 14:

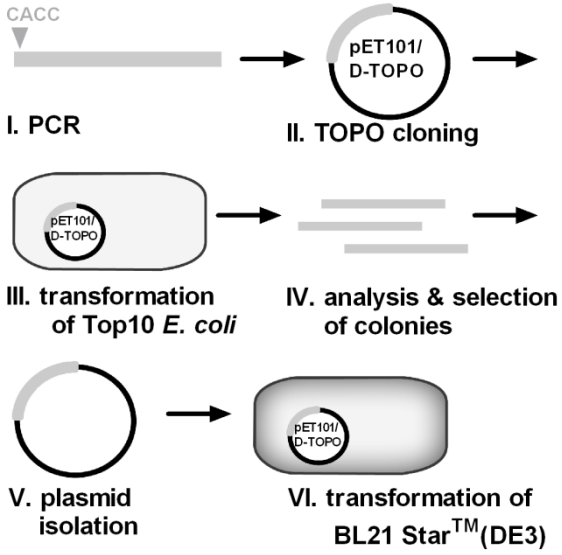

FIG. 14: Cloning strategy of SAK and Sbi 1-2.
(I) Blunt-end PCR products containing *sak* or *sbi 1-2* were amplified. (II) PCR products were inserted into the expression vector pET101/D-TOPO (III) and the resulting vectors were transformed into TOP10 E. coli cells. (IV) Colonies were analyzed by colony PCR. (V) Plasmid DNA was isolated from a positive transformant and sequenced. (VI) The desired plasmids were transformed into the expression strain BL21 Star.

PCR

A blunt-end PCR product of *sak* or *sbi 1-2* was amplified (GeneAmp® PCR System 9700-Applied Biosystems) using the appropriate primers, heat inactivated *S. aureus* Newman DNA as template, Phusion DNA polymerase (Finnzymes), and the following temperature cycle:

98°C 30s

35 cycles of: 98°C 20 s, 60°C 30 s, 72°C 30 s

72°C 7 min.

Agarose gel electrophoresis

Agarose gel electrophoresis was used to analyze the amplified DNA fragments. Samples were mixed with 6x DNA loading dye solution (Fermentas) and separated using 1% TBE (890 mM tris, 890 mM boric acid, 20 mM EDTA) agarose gel containing 0.1 µg/ml ethidium bromide. SmartLadder (Erogentec) was used as marker. DNA bands were visualized under UV light in a gel documentation system (BioRad).

TOPO cloning reaction and transformation

For the TOPO cloning reaction 2 µl of successfully amplified *sak* or *sbi 1-2* PCR reaction were mixed with 1 µl salt solution, 2 µl sterile water, and 1 µl TOPO vector and incubated for 5 min before the reaction mixture was transformed into One Shot TOP10 *E. coli* competent cells. Transformed bacteria were then cultivated on LB plates containing 50 µg/ml ampicillin.

Colony screening

Colonies were analyzed for positive clones using colony PCR with 2 µl of heat inactivated cell lysate, 1.5 µl of the appropriate forward and reverse primers, 7.5 µl sterile water, and 12.5 µl Hot Start Master Mix (Quiagen) in the following temperature cycle:

95°C 15 min

25 cycles of: 94°C 30 s, 60°C 40 s, 72°C 60 s

72°C 10 min.

Plasmid isolation & sequencing

PCR products were analyzed by agarose gel electrophoresis and *sak* or *sbi 1-2* containing clones were cultivated for plasmid isolation in 3 ml LB medium (supplemented with ampicillin) overnight at 37°C with shaking. Plasmids were isolated using Spin Plasmid Mini Two Kit (Invitek) following the manufacturer's instructions. Briefly, cells were harvested, resuspended in 250 µl of the supplied resuspension buffer, and lysed by lysing buffer. Thereafter a neutralization buffer was added and the mixture was centrifuged at 3000 g for 10 min. The supernatant was applied to a QIAprep spin column and the plasmid DNA was bound to the column through centrifugation at 3000x *g* for 30 s. Before elution of the plasmid DNA in 30 µl water, the column was washed with wash buffer.

In order to confirm that *sak* or *sbi 1-2* was in frame with the C-terminal His-tag and to exclude mutations in the sequence, plasmids containing the construct were sequenced via Sanger sequencing using an ABI PRISM® 3130x Genetic Analyzer (Applied Biosystem).

Storage of strains

For long term storage, glycerol stocks of the *E. coli* strains containing the desired pET constructs were prepared. Therefore 750 µl of an overnight culture was mixed with 250 µl sterile glycerol and frozen at -80°C.

Transformation of BL21 Star (DE3)

Plasmids containing the correct *sak* or *sbi 1-2* sequence were used to transform BL21 Star (DE3) cells. Therefore, 10 ng plasmid DNA (in 2 µl water) were added to thawed BL21 Star cells and incubated for 30 min on ice. After heat-shock, the cells were incubated in SOC medium (Invitrogen) and plated on LB plates containing ampicillin.

2.7 Protein Methods

(Glycine-) SDS-PAGE

Protein mixtures were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (124) using a Biometra system. Protein samples were diluted in loading buffer (0.31 M tris/base pH 6.8, 5% SDS, 50% glycerol, 0.001% bromphenol blue) or denatured by β-mercaptoethanol-containing Roti®-Load 1 (Roth) at 95°C for 5 min. Electrophoresis was performed using 4% stacking gels (3.0 ml distilled water, 1.25 ml 0.5 M tris/HCl pH 6.8 plus 0.4% SDS, 0.650 ml acrylamide-bisacryamide 30%, 25 µl 10% ammonium persulfate (APS), 5 µl N,N,N',N'- tetramethylendiamine (TEMED)) and 8% or 12% running gels (for 12%: 5.25 ml distilled water, 5.25 ml 1.5 M tris/base pH 8.8 plus 0.4% SDS, 5.0 ml acrylamide-bisacryamide 30%, 50 µl 10% APS, 10 µl TEMED) in running buffer (3% tris/base, 14.4% glycine, 1% SDS).

Tricine-SDS-PAGE

For resolving low molecular weight proteins and peptides, Tris-Tricine acrylamide gels were used. 4% stacking gels (0.81 ml acrylamide-bisacryamide 40%, 1.55 ml 3 M tris/HCl pH 8.4 plus 0.3% SDS, 3.89 ml distilled water, 40 µl 10% APS, and 15 µl TEMED) and 10% running gels (4.9 ml acrylamide-bisacryamide 40%, 5.0 ml 3 M tris/HCl pH 8.4 plus 0.3%, 1.15 ml distilled water, 3.95 ml 40% glycerol, 50 µl 10% APS, and 10 µl TEMED) were prepared using a Biometra system. The samples were electrophoresed at 80 V using anode buffer (0.2 M tris/HCl pH 8.9) and cathode buffer (0.1 M tris/HCl, 0.1 M tricine, 0.01% SDS).

Silver staining

To detect proteins after protein electrophoresis, silver staining was performed. Gels were fixed in 30% acetic acid plus 20% ethanol for 45 min, washed twice with 20% ethanol, and sensitized in 0.02% sodium thiosulfate for 2 min. The gels were then rinsed twice for 1 min in distilled water, stained for 20 min with 0.2% silver nitrate, and rinsed twice in distilled water again. Gels were developed using developer (0.00007% formaldehyde (37%), 3% sodium carbonate, 0.001% sodium thiosulfate), and the reaction was stopped by applying stop solution (2.5% acetic acid, 50% tris/base).

Coomassie staining

Coomassie staining (Page Blue, Fermantas) was performed according to the manufacturer's instructions.

Western blotting

To detect specific proteins after SDS-PAGE, Western blotting was performed. Gels were transferred to a nitrocellulose membrane (Protran) by placing them in the transfer cassette, immersed in transfer buffer (0.045 M tris, 0.039 mM glycine, 20% methanol, 0.1% SDS), and run at 48 mA for 70 min. Membranes were then blocked with blocking buffer (1% BSA, 4% milk powder, 0.1% Tween20) for 1 h at room temperature or overnight at 4°C, and incubated with appropriate primary and secondary antibodies (Ab) for 1 h at room temperature, washed with wash buffer (0.05% Tween in phosphate buffered saline (PBS) II (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.46 mM KH_2PO_4)), and developed with ECL-substrate solution (AppliChem) with a DNR BioImaging System (MF-ChemBIS 3.2).

Proteolytic generation of peptides derived from apoE

ApoE (0.5 µg) was incubated with 0.5–100% PMN lysate (from freeze/thaw disrupted polymorphonuclear neutrophils 10^6 cells/ml) or HNE (20 ng) at 37°C for 20 min. Samples were analyzed under nonreducing conditions using Tricine gels. Proteins were stained by Coomassie or transferred to nitrocellulose membranes. After blocking for 1 h at room temperature, membranes were incubated with anti-apoE polyclonal Ab (1:5000; Calbiochem), and the subsequent horseradish peroxidase (HRP)-coupled secondary Ab (1:5000; DAKO). Additionally, apoE (5 µg) was incubated with HNE (50 ng) for 5; 10 and 20 min. To assay the cleavage of the apoE-derived peptides LRV30 or SHL14, 25 µM peptide was incubated with 0.7 µM HNE for 1 h at 37°C. To analyze time- and

dose-dependency of HNE-mediated cleavage of LRV30, 25 µM LRV30 was incubated with 0.34 µM HNE for 0.5, 1, and 5 h, or 25 µM LRV30 was incubated with 0.034–3.4 µM HNE for 1 h. All samples were separated using Tricine gels and proteins were stained with Coomassie.

Plasmin-mediated C3/C3b degradation

To characterize C3 cleavage products generated by plasmin-mediated C3, C3b, iC3b or C3c degradation, fluid phase assays were performed. 15 µg/ml PLG was mixed with 2 µg/ml uPa, 7 µg/ml C3 (or C3 fragment), and incubated for 3 h at 37°C. In order to compare plasmin- and factor I-mediated cleavage of C3 fragments, 10 µg/ml PL or 5 µg/ml factor I plus 10 µg/ml factor H were incubated with 7 µg/ml C3, C3b or iC3b for 1 h at 37°C. Protein mixtures were separated by SDS-PAGE and C3 cleavage products were analyzed by Western blotting using anti-C3 pAb.

To assess C3 or C3b degradation within a complex of plasmin and Sbi or Efb, 0.2 µM PLG together with either 0.04 µM C3 or C3b were added to immobilized Sbi, Efb, and the control proteins CRASP-5 or HSA overnight at 4°C. After extensive washing, 0.5 µM SAK or 0.06 µM uPa were added for 3 h at 37°C. Samples were reduced with Roti-Load for 5 min at 95°C, separated by SDS-PAGE, and transferred onto a nitrocellulose membrane. C3/C3b and their cleavage products were detected with anti-C3-Fab-HRP (1:1000).

The effect of Sbi, Efb or their truncated constructs on the plasmin-mediated C3 degradation was analyzed by incubating 0.04 µM C3, C3b or iC3b with either 0.15 µM PLG plus 0.04 µM uPa or 0.2 µM PLG plus 0.5 µM SAK in the absence or presence of 1 µM Sbi, Efb, CRASP-5 or HSA for 3 h at 37°C. Additionally, the same assays were performed using 0.2-2 µM Sbi 1-2, Sbi 3-4 or Efb-C instead of the Sbi or Efb. To assess the degradation-enhancing effect of Efb-C on aureolysin-mediated cleavage, 2 µM Efb-C was incubated with either 0.15 µM aureolysin or plasmin, and 0.04 µM C3 or C3b, for 3 h at 37°C. Samples were analyzed by SDS-PAGE and Western blotting using anti-C3-Fab-HRP (1:1000).

Degradation of apoE and SHL14 by staphylococcal proteases

To test the proteolytic activity of aureolysin and plasmin against SHL14, 25 µM SHL14 was incubated with the indicated amounts of aureolysin or plasmin (FIG. 24) for 10 min. Additionally, SHL14 was mixed with 0.22 µM plasminogen plus 0.6 µM SAK for 0.5 h and 1 h. In order to assess the degradation of apoE by aureolysin and plasmin, 3 µM

apoE was incubated with 0.5 µM aureolysin or 0.22 µM plasminogen plus 0.6 µM SAK for 0.5 h and 1 h. Furthermore, the degradation of apoE by different concentrations of aureolysin or plasmin (indicated in Fig.6 B) was assessed for 30 min. All samples were separated using Tricine gels and proteins were stained with Coomassie.

Ligand blot

To analyze binding of Sbi or Efb to plasminogen and to verify the accuracy of the recombinant staphylococcal proteins, Sbi and Efb were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking for 1 h at room temperature, the membranes was incubated with 1 µg/ml biotin-coupled plasminogen (PLG_b) or C3 overnight at 4°C. Bound PLG_b was detected with Streptavidin-Peroxidase (1:1000; Sigma), C3 with anti-C3-Fab-HRP (1:1000; Protos Immunoresearch), and to assess IgG-binding, anti-goat-HRP (1:1000; DAKO) was applied. All Western blots were developed as described above.

Surface plasmon resonance (SPR)

Plasminogen binding to Sbi 3-4 or Efb-C was analyzed in real time by surface plasmon resonance (Biacore 3000, AB) at 25°C in 150 mM PBS. Sbi 3-4 or Efb-C was immobilized on a CMD 500M sensor chip (Xantec) by standard amino coupling chemistry following the manufacturer´s protocol. PLG (400 nM) was injected at a flow rate of 5 µl/min.

Enzyme Linked Immunosorbent Assay (ELISA)

To assess the binding interactions of Sbi, Efb, and their fragments to plasminogen, ELISAs were performed. Bacterial proteins were immobilized (equimolar) on a microtiter plate (Maxisorb, Nunc) blocked with 0.4% gelatin in DPBS for 2 h at 37°C. PLG_b was added for 1.5 h at 37°C and bound proteins were detected with Streptavidin-peroxidase (1:1000) for 1 h. The reaction was developed with TMB (KEM EM TEC), stopped by addition of 0.25 M H_2SO_4, and measured at 450 nm (SpektraMAX 190, Molecular Devices).

For dose-dependent binding of PLG to Sbi 3-4/Efb-C, 5 µg/ml Sbi 3-4 or Efb-C was immobilized on a microtiter plate, blocked with Blocking Solution I (AppliChem) for 2 h at 37°C, and 25-200 nM PLG was added for 1.5 h at 37°C. Bound proteins were detected with anti-PLG Ab (Acris Antibodies; 1:1000) in Cross Down Buffer (AppliChem) and anti-goat-HRP (1:2000).

In competition assays, Sbi 3-4 or Efb-C was immobilized, and incubated with C3 and PLG (molar ratio: 1:0; 1:0.5, 1:1, 1:4, and 0:1). C3 binding was detected using polyclonal anti-C3 Ab (Comptech; 1:2000) and PLG binding with anti-PLG Ab.

To analyze the effect of the truncated Sbi 3-4 or Efb-C on the plasmin-mediated C3 degradation, C3 ELISAs were performed. C3 (0.04 µM) was immobilized on a microtiter plate at room temperature, after blocking with 0.4% gelatin, 0.15 µM PLG with 0.04 µM uPa or 0.2 µM PLG with 0.5 µM SAK, was incubated for 3 h at 37°C. Sbi 3-4, Efb-C, CRASP-5, or HSA (1 µM each) was added in the presence or absence of plasmin. After extensive washing, bound C3 was detected using anti-C3a Ab supplemented with 15 µg/ml plasmin inhibitor aprotinin and then HRP-coupled Ab at 4°C.

Combined ELISA-Western blot Assay (CEWA)

Binding of Sbi, Efb, and SAK to plasminogen was analyzed by combining ELISA and Western blotting. CEWA was performed according to (90). Briefly, bacterial proteins, HSA or buffer were immobilized on a microtiter plate. After blocking with gelatin, 50 nM plasma purified PLG was added and incubated overnight at 4°C. Bound PLG was eluted using SDS buffer, separated by SDS-PAGE, and analyzed by Western blotting using anti-PLG Ab. *Borrelia burgdorferi* CRASP-5 protein was included as a positive control and HSA as a negative control.

Protein expression & purification

All recombinant staphylococcal proteins were expressed as shown in FIG. 15:

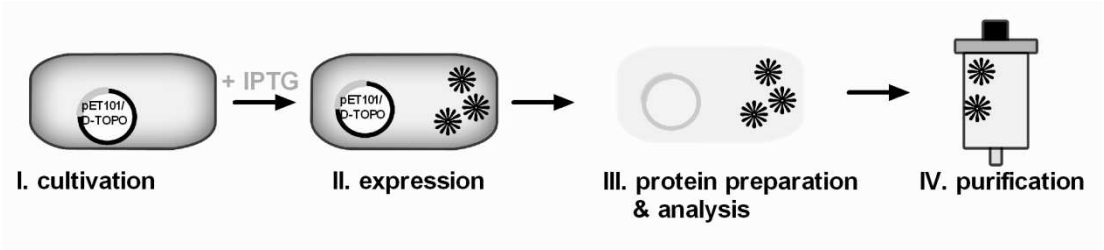

FIG. 15: Expression and purification of SAK and Sbi 1-2.
(I) *E. coli* BL21 Star containing the expression plasmid was cultivated to mid-log. (II) IPTG was then added to induce protein expression. (III) Cells containing the desired protein were harvested by centrifugation and lysed. (IV) The supernatants were diluted in binding buffer and loaded onto a nickel column. After washing, proteins were eluted from the column by imidazole. Elute fractions were concentrated, and buffer was exchanged to DPBS.

200 ml LB medium were inoculated with 10 ml overnight culture and the bacteria were grown to mid-logarithmic phase, before expression of the desired staphylococcal protein was induced by 1 mM isopropyl-b-D-thiogalactoside (IPTG) for 3 h. Cells were

harvested and frozen at -20°C. The cell pellet was resuspended in Lysis Buffer (50 mM potassium phosphate, pH 7.8, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, 10 mM imidazole), and lysed by repeated freezing and thawing.

The cell lysate was centrifuged, the cleared supernatant was diluted in buffer A (10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 0.5 M NaCl, 10 mM imidazole), and loaded onto a 1 ml HisTrap™ HP column (GE Healthcare). The column was washed with buffer A and 5% buffer B (10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 0.5 M NaCl, 0.5 M imidazole) using an AktaPurifier 10 chromatography unit (GE Healthcare). SAK or Sbi 1-2 was eluted from the column by 100% buffer B. Elute fractions were concentrated using centrifugal filter units (Millipore) and buffer was exchanged to DPBS. Protein concentration of purified SAK or Sbi 1-2 was determined by NanoDrop. The purity was analyzed by SDS-PAGE and silver staining or Western blotting.

The borrelial CRASP-5 protein was kindly provided by Prof. Dr. P. Kraiczy.

Biotinylation

ApoE and PLG were biotinylated with Sulfo-NHS-LC-Biotin (Thermo Scientific) according to manufacturer´s instructions. However, a 10 fold molar excess of biotin was used. Unbound biotin was removed by Zeba™ Desalt Spin Columns (Pierce).

Plasmin activity assay

PL activation was analyzed by hydrolysis of the PL-specific substrate S-2251 (D-valyl-leucyl-lysine-ρ-nitroanilide dihydrochloride; Haemochrom Diagnostica). To determine functional activity of recombinant SAK, 10 µg/ml PLG was immobilized onto a microtiter plate overnight, blocked with 1% bovine serum albumine (BSA) in DPBS, and PLG was activated using either 0.08 µg/ml uPa or 1 µg/ml recombinant SAK together with the chromogenic substrate S-2251 dissolved in reaction buffer (64 mM tris, 350 mM NaCl, 0.01% triton-X; pH 7.5). PL activity was recorded at 1 h intervals at 405 nm (SpektraMax 190; Molecular Devices).

The accessibility of Sbi- or Efb-bound PLG for activation by SAK or uPa was assayed by immobilizing equimolar amounts of Sbi, Efb, and the control proteins CRASP-5 and HSA onto a microtiter plate. After blocking with 0.4% gelatin, 0.2 µM PLG were incubated at 4°C overnight. Following washing with PBS+0.05% Tween20, uPa (0.08 µg/ml) or recombinant SAK (1 µg/ml) was added together with the chromogenic substrate S-2251 dissolved in reaction buffer. PL activity was recorded at 4 h intervals at 405 nm.

Fibrinogen degradation assay

PL activation by SAK was further analyzed by degradation of fibrinogen. 20 µg/ml PLG, 1 µg/ml SAK, and 20 µg/ml fibrinogen were incubated for 24 h at 37°C. Samples were reduced with RotiLoad (Roth), separated by SDS-PAGE, and transferred to a nitrocellulose membrane. Fibrinogen degradation fragments were identified using anti-fibrinogen Ab (Calbiochem) and anti-rabbit Ab (DAKO).

2.8 Microbiological assays

Antibacterial assays

Viable Count Analysis: *S. aureus* Newman or *E. coli* DH5α was grown to mid-logarithmic phase in LB medium. Bacteria were washed, diluted in 10 mM tris buffer supplemented with 5 mM glucose, and 10^4 CFUs were incubated with 3 µM apoE plus 20-29 nM HNE with 1 µM or the indicated amounts (FIG. 17E) of LGR11; SHL14 or WGE23. To assess whether heparin-binding sites are essential for the bactericidal activity, 10 µg heparin were added to the samples. Bacteria were then diluted in tris buffer and plated on LB agar, incubated overnight at 37°C, and colony-forming units (CFUs) were determined. 100% survival was defined as total survival of bacteria in the same buffer as in the absence of HNE or the peptides.

To assay the impact of plasmin on C3a antistaphylococcal activity, 10^4 CFUs of *S. aureus* were incubated with 2 µg C3a alone or with 5 µg PLG and/or 1 µg SAK for 2 h at 37°C. Samples were analyzed as described for apoE-derived peptides. 100% survival was defined as total survival of bacteria in the same buffer as in the absence of C3a. In a parallel setting, supernatants were separated by SDS-PAGE and analyzed by Western blotting with anti-C3a Ab (1:1000 Comptech).

Viable OD Analysis: The antibacterial effects of the apoE-derived peptides were further confirmed by viable OD analysis. Samples were prepared as described for the viable count analysis, however after 2 h incubation the bacteria were diluted in TSB medium and transferred to a microtiter plate (Greiner). All samples were measured in triplicate at OD_{650} over 14 h at 37°C (SpektraMax 190; Molecular Devices). To evaluate the impact of aureolysin or plasmin on SHL14 bactericidal activity, *S. aureus* was incubated with 2 µM SHL14 together with the indicated amounts (FIG. 25 B) of aureolysin or plasmin, and viable OD analysis was performed.

Fluorescence Microscopy

S. aureus was grown to mid-logarithmic phase in LB medium. Bacteria were washed and diluted in 10 mM tris buffer. 2.5 x 10^7 CFUs were incubated with 40 µM FITC-coupled SHL14 for 30 min on ice. To assess aureolysin- or plasmin-mediated degradation of SHL14, 0.33 µM aureolysin, 0.25 µM plasmin or 0.30 µM HNE was added together with SHL14-FITC to the bacteria at 20°C. All samples were stained with wheat germ agglutinin (WGA)-Texas Red (Invitrogen), and then washed twice with tris buffer at 4°C. Stained bacteria were applied onto microscope slides (Roth) and dried for 2 h at 4°C. The cover glass (VWR) was mounted on a slide using Mount Fluor (Pro Taqs). *S. aureus* was visualized using LSM 710 (Zeiss).

Flow Cytometry

The interaction between apoE and the staphylococcal surface was analyzed using flow cytometry. *S. aureus* was washed and incubated with 0.5-2.5 µg biotin-labeled apoE for 1 h at 37°C. To analyze the impact of aureolysin or plasmin on apoE deposition, increasing amounts of aureolysin and plasmin (2.5-10 µg/ml) were incubated together with 1.5 µg biotinylated apoE for 1 h at 37°C. After extensive washing, streptavidin-Cy5 (Invitrogen) was added for 30 min at 4°C. Following washing, bacteria were analyzed by flow cytometry using LSR II flow cytometer (BD). All incubation and washing steps were performed in PBS II supplemented with 1% BSA.

The effects of plasmin and Sbi or Efb on C3b deposition were analyzed on the staphylococcal surface. *S. aureus* was heat inactivated for 15 min at 72°C, washed with PBS II containing 1% BSA and incubated with 5% serum in HEPES buffer (20 mM Hepes, 140 mM $NaCl_2$, 5 mM $CaCl_2$, 25 mM $MgCl_2$; pH 7.4) for 20 min at 37°C. After washing, bacteria were incubated with 2 µM Sbi 1-2, Sbi 3-4, Efb-C or HSA in the absence or presence of 0.15 µM PL (Calbiochem) for 2 h at 37°C. Supernatants were reduced with Roti-Load, separated by SDS-PAGE, and analyzed by Western blotting using anti-C3-HRP. Bacteria were washed twice and FITC-labeled anti-C3 Fab (Protos Immunoresearch) was added for 20 min at 4°C. Following washing, surface-bound C3b was measured by flow cytometry.

ApoE/Plasma Absorption Assay

S. aureus was incubated with 1 µg apoE or 15% normal human plasma (NHP) in PBS II with 1% BSA for 1 h at 37˚C. After extensive washing, bacteria were treated with elution buffer (60 mM tris, 2% SDS, 25% glycerin) for 10 min at 37˚C. The supernatants were

separated by SDS-PAGE and transferred to a membrane. ApoE was identified by Western blot analysis with anti-apoE pAb together with an appropriate HRP-goat-antiserum.

Adhesion & Phagocytosis Assay

S. aureus was incubated with or without 0.6-1.8 µM apoE for 30 min at 37°C, washed, and stained with DiD (Invitrogen). The impact of aureolysin or plasmin on apoE-mediated phagocytosis was assayed by incubating *S. aureus* with 10 µg/ml aureolysin or plasmin and 60 µg/ml apoE. THP-1 cells, stimulated to become macrophages by 4 µg/ml phorbol 12-myristate 13-acetate (PMA), were stained with DiO (Invitrogen). DiD-stained bacteria were added to the DiO-stained macrophages for 150 min. Free bacteria were removed by washing and the macrophages were then detached using 0.25% trypsin/ 0.05% EDTA solution (Biochrom). The extent of adhesion and phagocytosis of *S. aureus* by macrophages was quantified as DiO/DiD double positive macrophages using flow cytometry.

2.9 Bioinformatical databases

Protein Knowledgebase (Uniprot) (www.uniprot.org), RCBS Protein Data Bank (www.pdb.org), and the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) were used to obtain structure and sequence information.

2.10 Statistical analyses

Significant differences between two groups were analyzed by the unpaired Student's t-test (www.graphpad.com). Values of $*p<0.05$, $**p<0.01$, $***p<0.001$ were considered as statistically significant.

3 RESULTS

3.1 ApoE has antimicrobial and opsonic activities

The lipoprotein transporter apoE is associated with cardiovascular disorders and the Alzheimer's disease (64, 66). More recently, immunoregulatory functions of apoE have been discussed (69, 70, 72, 73). Thus, the aim of this study was to characterize the role of apoE in the innate immune system.

3.1.1 ApoE is degraded by HNE into AMPs

ApoE contains several amphipathic α-helixes (64). Because these structures are a common feature of AMPs, synthetic peptides of several plasma proteins, including apoE, were generated and reported to exert antimicrobial activity (74, 76). However, the natural appearance and generation of apoE-derived AMPs remained elusive. First, we assayed whether native apoE acts antibacterial against *S. aureus* and *E. coli*. Therefore bacteria were incubated with apoE and colony forming units (CFUs) were determined. ApoE had no bactericidal effect (data not shown).

Next, the antibacterial activity of apoE after proteolytic processing was investigated. Polymorphonuclear neutrophils (PMNs) are largely recruited to infectious sites and subsequently release proteases, such as human neutrophil elastase (HNE), cathepsin G, and proteinase 3 (125, 126). Thus, intact apoE was incubated with lysates of PMNs or purified HNE, and cleavage of apoE was analyzed using Tricine-SDS-PAGE followed by Western blotting or Coomassie staining. PMN-lysate and purified HNE degraded apoE into similar apoE fragments ranging from 4.6 to 28 kDa (FIG. 16 A+B).Predominantly low-molecular weight fragments were detected after 20 min.

To investigate the bactericidal effects of these apoE-derived peptides, bacterial survival was analyzed in viable count assays. *S. aureus* or *E. coli* was incubated with apoE and HNE, plated on LB plates, and CFUs were determined (FIG. 16 C). HNE-cleaved apoE, compared to the intact protein, decreased bacterial survival of *S. aureus* and *E. coli* (black columns). In addition, HNE alone exhibit minor bactericidal activity (white columns). However, in the presence of apoE and HNE, survival of *S. aureus* decreased about 64% and survival of *E. coli* about 51% compared to HNE alone, thus apoE-derived AMPs were generated.

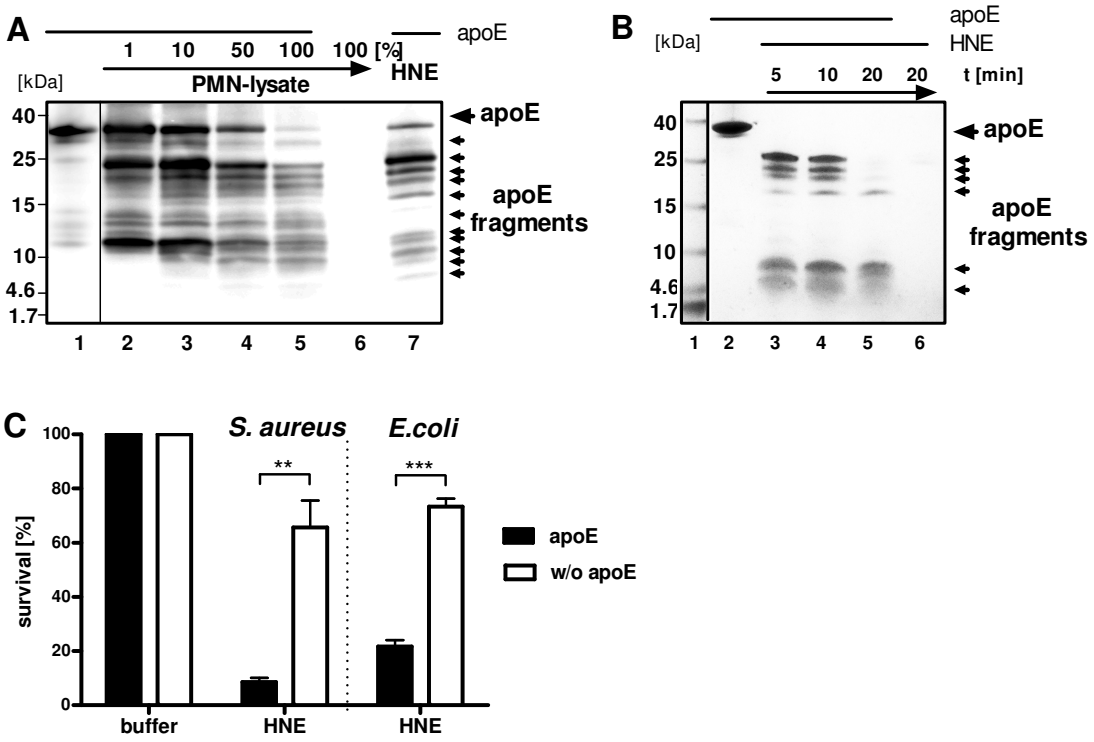

FIG. 16: HNE cleaves apoE and generates antibacterial peptides.

(A) Degradation of apoE by PMN lysates or HNE yielded similar apoE fragments (indicated by arrows). ApoE was incubated with polymorphonuclear neutrophil (PMN) lysate or human neutrophil elastase (HNE), samples were analyzed by Tricine-SDS-PAGE and Western blotting using anti-apoE Ab. (B) ApoE was degraded by HNE into low-molecular weight fragments. ApoE was incubated with HNE; samples were drawn at the indicated time points, separated by SDS-PAGE, and stained with Coomassie. (C) HNE-cleaved apoE decreased bacterial survival. *S. aureus* or *E. coli* was incubated with apoE and HNE under low salt conditions. Bacteria were plated on LB plates, incubated overnight, and CFUs were determined. Total survival of bacteria without HNE treatment was defined as 100% survival. HNE alone exhibited also bactericidal activity. Data shown are mean values of at least three independent experiments; error bars indicate standard deviations. ** $p < 0.01$ and *** $p < 0.001$.

3.1.2 ApoE-derived AMPs encompass a heparin-binding site

Synthetic peptides derived from the heparin-binding sites of human plasma proteins have been shown to exert antimicrobial activity (9, 11, 127). To elucidate whether HNE-mediated cleavage of apoE generates AMPs containing heparin-binding sites, heparin was added to bacteria treated with HNE-cleaved apoE and viable count assays were performed. Heparin inhibited the bactericidal activity of the HNE-generated apoE fragments against *S. aureus by* ~78% and against *E. coli* by ~75% (FIG. 17 A). By contrast, heparin alone exerted only minor effects on HNE activity. Consequently, apoE-derived AMPs encompassed heparin-binding motifs.

To investigate which of the two heparin-binding sites in apoE contributes to antimicrobial activity, apoE-derived peptides SHL14 and WGE23 were

synthesized, containing one of the two heparin-binding sites, flanked by cleavage sites for HNE. A fragment encompassing a part of the N-terminal apoE domain without a heparin-binding site (LGR11) was included as a negative control (FIG. 17 B). The antibacterial activities of LGR11, SHL14, and WGE23 were assessed by adding the peptides to bacteria, and measuring bacterial growth at OD_{650} over 14 h. SHL14, which partly contains the LDL-receptor-binding region, inhibited multiplication of both, *S. aureus* (FIG. 17 C) and *E. coli* (not shown). By contrast, WGE23 and LGR11 exhibited no antibacterial activity. These results were confirmed by viable count assays (FIG. 17 D). SHL14 antibacterial activity against *S. aureus* was abolished by the addition of heparin (FIG. 17 D). In addition, SHL14 dose-dependently decreased survival of *S. aureus* and *E. coli* (FIG. 17 E). Thus, bactericidal activity of apoE is located in the N-terminal heparin-binding site.

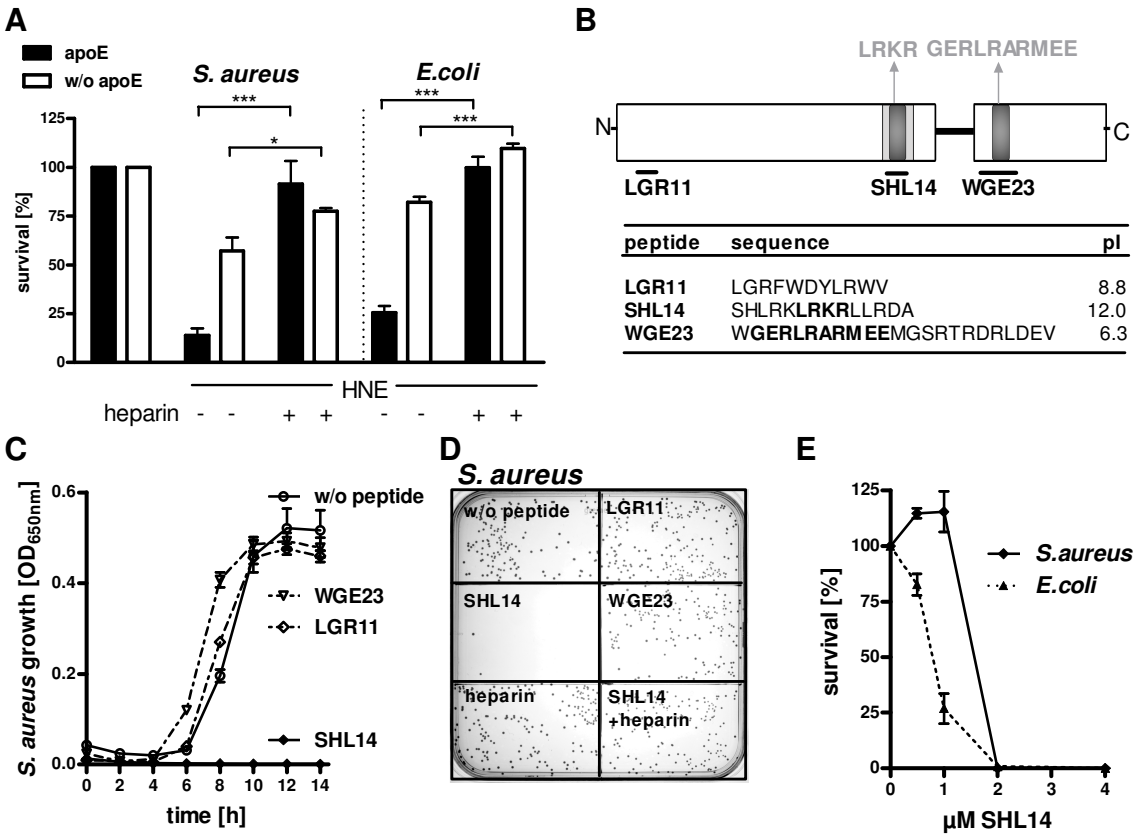

FIG. 17: ApoE-derived AMPs contain a heparin-binding site.
(A) Heparin inhibited antibacterial activity of HNE-generated apoE fragments. *S. aureus* or *E. coli* was incubated with apoE and HNE in the presence or absence of heparin, and antibacterial activity was determined by viable count assays. (B) Scheme of apoE indicating the positions, sequences, and isoelectric points (pI) of the synthetic peptides. SHL14 and WGE23 contain a heparin-binding site (labeled in bold letters) and are flanked by two potential HNE cleavage sites. LGR11 was included as negative control. (C+D) SHL14 acts bactericidal against *S. aureus*. Antibacterial activity of LGR11, SHL14, and WGE23 were tested in antibacterial assays. WGE23 and LGR11 had no bactericidal effect. (D) The bactericidal activity of SHL14 was inhibited by the addition of heparin. Dots

represent single colonies of *S. aureus* plated on agar plates. (E) SHL14 killed *S. aureus* and *E. coli* in a dose-dependent manner. The indicated amounts of SHL14 were incubated with *S. aureus* or *E. coli*, and viable count assays were performed. (A+C) Data represent mean values of at least three independent experiments. Error bars indicate standard deviations $*p < 0.05$ and $***p < 0.001$. (D+E) Data shown are representative for three independent experiments.

3.1.3 SHL14 is essential for antibacterial activity

During the cleavage of apoE by HNE, multiple peptides containing the SHL14 sequence are presumably generated (FIG. 18 A). Thus, we assayed whether the longer peptide LRV30, which was described by Azuma *et al.* (74), can be further degraded by HNE. LRV30 or SHL14 was incubated with HNE, cleavage products were separated by Tricine-SDS-PAGE, and stained using Coomassie. LRV30 (~4 kDa) was further degraded into smaller fragments of ~3 and ~2 kDa. However, SHL14 (~2 kDa) was not cleaved (FIG. 18 B). In parallel, the dose- and time-dependency of HNE-mediated degradation of LRV30 was assessed. LRV30 was cleaved into fragments of similar mobility to SHL14 (FIG. 18 B+C). Hence, SHL14 is likely to be generated by HNE-mediated cleavage of apoE and is included in all antibacterial peptides derived from apoE. Thus, the activity of SHL14 was characterized in more detail.

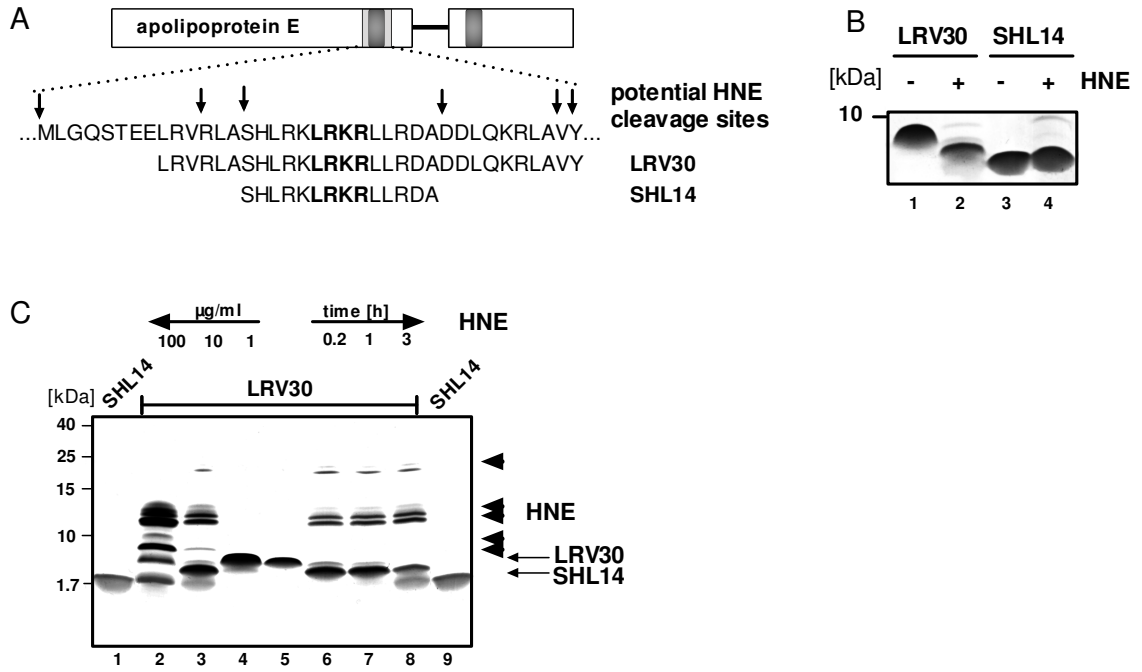

FIG. 18: SHL14 is essential for antibacterial activity.
(A) Part of the apoE sequence containing the N-terminal heparin-binding site (labeled in bold letters). Potential HNE cleavage sites (after valine and alanine) are shown by arrows. LRV30 contains several potential cleavage sites. (B) LRV30, but not SHL14, was further cleaved by HNE. LRV30 and SHL14 were incubated with HNE, separated by Tricine-SDS-

PAGE, and stained with Coomassie. (C) Dose- and time-dependent proteolysis of LRV30. Indicated amounts of HNE were used (lane 2-4) or samples were drawn at the indicated time points (lane 6-8). (B+C) A representative result out of three independent experiments is shown.

3.1.4 SHL14 binds to *S. aureus*

To investigate the interaction between SHL14 and *S. aureus,* fluorescence microscopy was performed. FITC-labeled SHL14 was incubated with Texas Red-stained bacteria and unbound peptides were removed by washing. SHL14 bound to the bacterial surface, as visualized by LSM (FIG. 19 A, panel I).

To localize the interaction of SHL14 with *S. aureus*, Z-stacking analysis was performed. The data revealed that SHL14 passed through the cell wall and presumably interacted with the cell membrane or intracellular targets (FIG. 19 B).

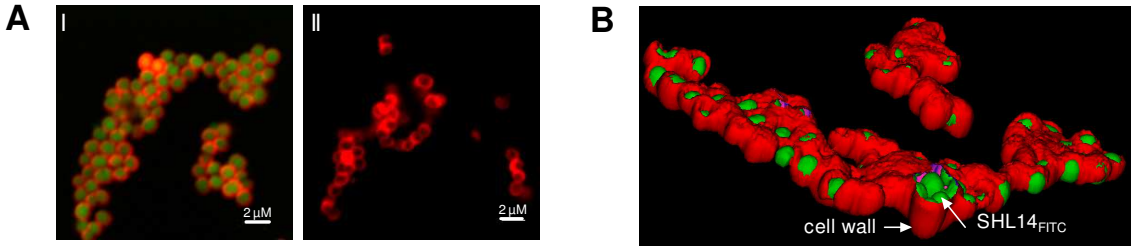

FIG. 19: SHL14 binds to *S. aureus.*
(A) Texas-Red stained *S. aureus* (red fluorescence) was incubated with (I) or without (II) FITC-labeled SHL14 (green fluorescence). After washing, bacteria were fixed and analyzed by fluorescence microscopy. (B) SHL14 passed through the cell wall. Z-Stacking was performed to localize SHL14. (A+B) Data shown are representative for three independent experiments.

3.1.5 Properties of SHL14

SHL14 was depicted in a helical wheel projection (FIG. 20 A) and a space-filling model (B) for structural characterization. Positive charged amino acids were colored in blue, negative charged in pink, and hydrophobic amino acids remained gray. SHL14 showed in both models clusters of hydrophilic and hydrophobic residues, demonstrating an amphipathic nature of the peptide. Furthermore, the amino acid residues of SHL14 were highlighted in the apoE protein structure model, showing that the peptide sequence is partially buried in the intact apoE (FIG. 20 C).

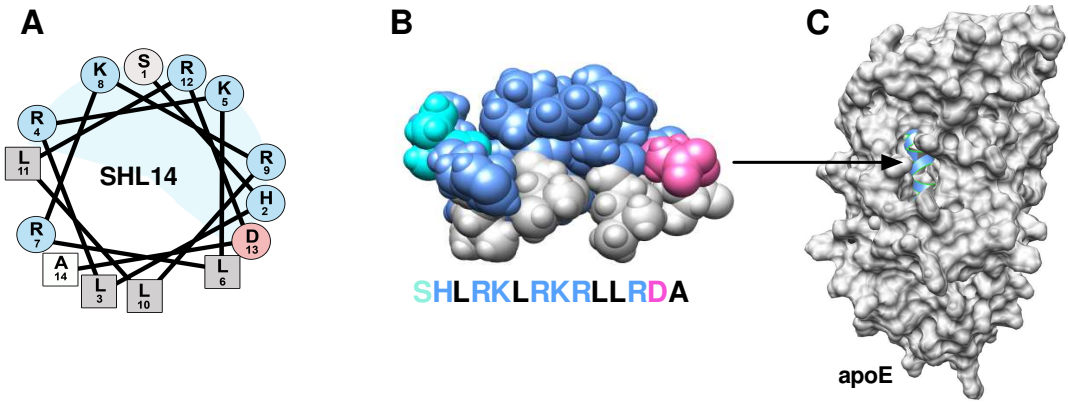

FIG. 20: Structure of SHL14.
(A+B) Helical wheel projection and space-filling model of SHL14 revealed an amphipathic peptide structure. Positive charged amino acid residues are shown in blue, negative charged in pink, and hydrophobic in grey. (C) SHL14 is partially buried in the interior of apoE. The backbone of SHL14 is illustrated in blue in the apoE surface model. (A) Modified after http://cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html. (B-C) Images were prepared with Chimera software using PDB file 2L7B (65).

3.1.6 ApoE bound to *S. aureus* enhances phagocytosis

To confirm the interaction of unprocessed apoE with the staphylococcal surfaces, flow cytometry was performed. Biotin-labeled apoE bound dose-dependently to *S. aureus* (FIG. 21 A). Furthermore, unlabeled apoE or normal human plasma was incubated with *S. aureus*, after extensive washing (wash), bound proteins were eluted (eluate), separated by SDS-PAGE, and apoE was detected by Western blot analysis. Purified apoE and apoE from NHP bound to *S. aureus* (FIG. 21 B).

Because apoE modulates uptake of apoptotic bodies (72) and promotes clearance of β-amyloid (73), we assessed whether apoE also influences the phagocytosis of bacteria. To test this hypothesis, apoE was deposited on *S. aureus*. Bacteria were stained with DID, and added to DIO-labeled THP1 macrophages, subsequently the ingested and adsorbed bacteria were measured by flow cytometry. ApoE increased the uptake and adhesion of bacteria up to 26.5%. This opsonic effect was dose-dependent. SHL14 did not influence phagocytosis, indicating that the effect of apoE was specific (FIG. 21 C+D).

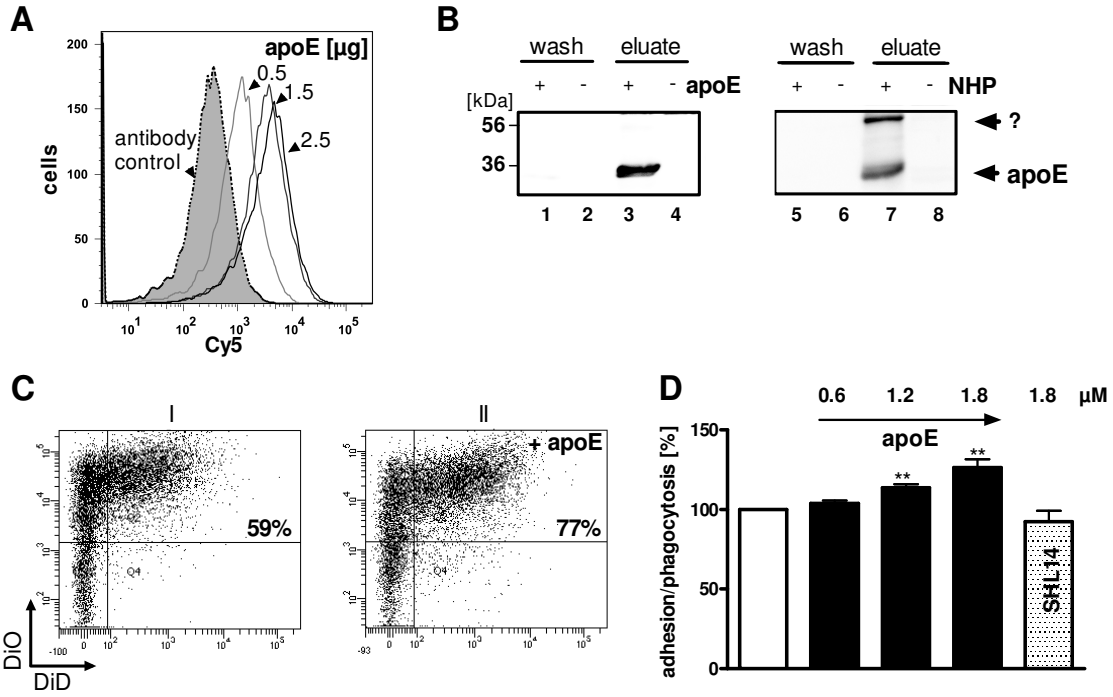

FIG. 21: Intact apoE binds to *S. aureus* and facilitates phagocytosis.
(A+B) ApoE bound to *S. aureus*. (A) Biotin-labeled apoE was mixed with intact bacteria. After washing, the amount of bound apoE was assayed by streptavidin-Cy5 using flow cytometry. (B) Nonlabeled apoE was incubated with *S. aureus*, and bound protein was eluted after extensive washing. Samples were analyzed by SDS-PAGE and Western blot using anti-apoE Ab. (C) ApoE enhanced adhesion and phagocytosis of *S. aureus* by human macrophages. *S. aureus* was incubated with buffer (I) or 1.8 µM apoE (II), stained with DiO, and added to DiD-labeled THP-1 macrophages. Macrophages with adherent and phagocytosed *S. aureus* were identified as DiO and DiD positive cells (2nd quadrant). (D) ApoE increased the adhesion/phagocytosis of *S. aureus* in a dose-dependent manner. Adhesion/phagocytosis of *S. aureus* without apoE was set to 100% (white bar). (A-C) Results are from one representative experiment out of three performed. (D) Values and error bars represent average and standard deviation of three independent experiments. $^{**}p < 0.01$.

3.2 Plasmin & aureolysin are used by *S. aureus* to evade apoE immune functions

S. aureus belongs to the most successful human pathogens owning to the vast arsenal of secreted immune evasion molecules. Among these factors are proteases and activators of host proteases (26, 128, 129). In this chapter the effects of both, aureolysin and the host-derived protease plasmin, which is activated by the staphylococcal plasminogen activator SAK, on the apoE-mediated immune functions were investigated.

3.2.1 Cloning, expression, and purification of SAK

To characterize SAK functions, the protein was recombinantly expressed in *E. coli*. The *sak* gene was amplified without the signal sequence, resulting in a 493 bp

PCR product (FIG. 22 A). This product was cloned into the pET101 vector, and transformed into *E. coli* Top10 cells. Six of the resulted colonies were screened for the desired insert using colony-PCR (FIG. 22 B). Plasmids containing a 493 bp insert were purified and sequenced. Sequencing showed that *sak* was correctly inserted into pET101. Plasmids were isolated and then used to transform the *E. coli* expression strain BL21 Star.

SAK expression was induced by IPTG and protein was purified by nickel-chelate affinity chromatography as described in chapter 2.7. The elution profile showed that protein was eluted from the column (blue line) after increase of imidazole concentration (brown line) (FIG. 22C). Recombinant expressed SAK was detected by SDS-PAGE, and visualized by silver staining (FIG. 22 D, lane 1) or Western blotting using anti-His Ab (lane 2). In both assays a clear band with a mobility of ~19 kDa was observed.

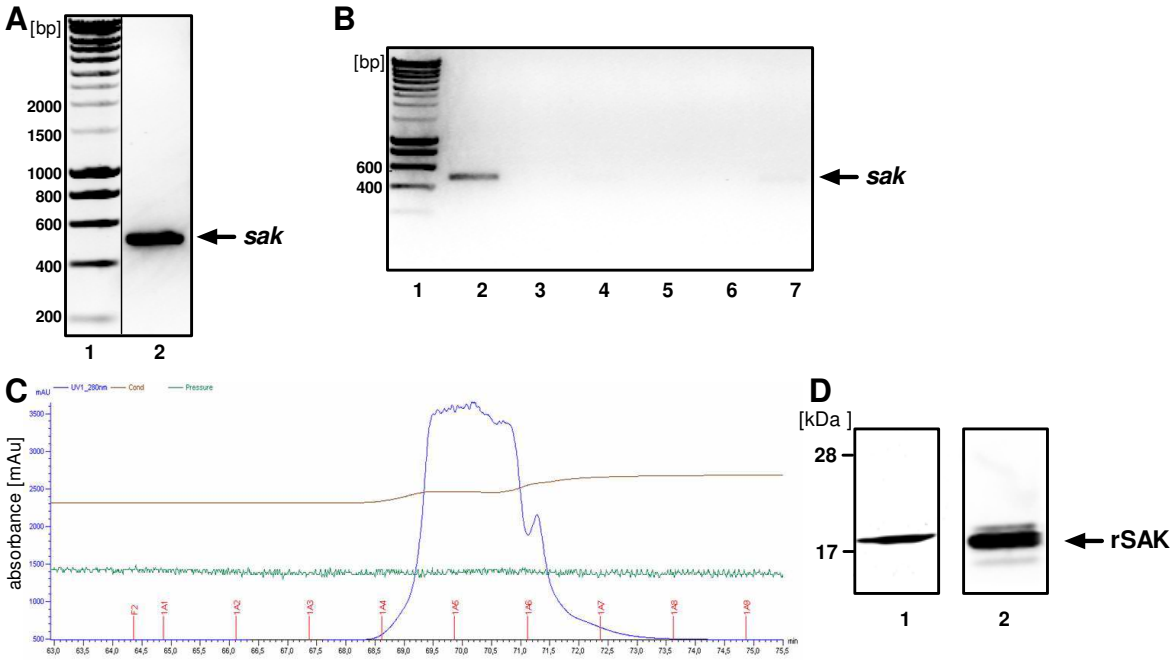

FIG. 22: Cloning, purification, and expression of SAK.
(A) *Sak* was amplified by PCR resulting in a 493 bp blunt-end product. (B) PCR screening for pET101 *sak* positive clones. The PCR product was inserted in pET101 and transformed into *E. coli* Top10 cells. Six clones were tested by colony-PCR. One positive clone was identified (lane 2). (C) Elution profile of SAK expression. Plasmids were transformed into BL21 Star and expression was induced by IPTG. Proteins were purified by IMAC. The blue line visualizes UV absorbance, which correlates with protein concentration and the brown line shows salt concentrations. (D) Recombinant SAK had a mobility of 19 kDa. Purified SAK was separated by SDS-PAGE and visualized by Western blotting using anti-HIS Ab (lane 1) or stained with silver (lane 2).

3.2.2 Functional characterization of recombinant SAK

To assess the functional activity of recombinant SAK, binding and activation of plasminogen was assayed. SAK bound to plasma purified plasminogen as detected by a distinct band with a mobility of 92 kDa in CEWA (FIG. 23 A). SAK-mediated conversion of plasminogen to plasmin was assayed by visualizing proteolysis of two plasmin-sensitive substrates. SAK activated plasminogen to plasmin, as shown by the cleavage of a chromogenic substrate (FIG. 23 B, solid lines), as well as the degradation of fibrinogen (FIG. 23 C), which was followed by SDS-PAGE and Western blot analysis using anti-fibrinogen Ab. However, a higher amount of SAK was needed to activate plasminogen compared to uPa.

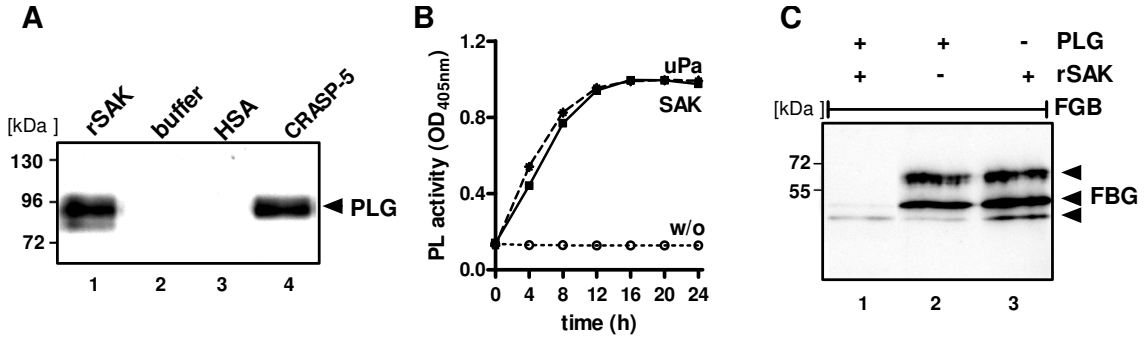

FIG. 23: SAK binds and activates plasminogen to plasmin.
(A) SAK recruited plasminogen (PLG). SAK, buffer, HSA, and borrelial plasminogen-binding protein CRASP-5 were immobilized to a microtiter plate and plasminogen was added. After extensive washing, bound plasminogen was eluted and visualized by SDS-PAGE and Western blotting. (B) SAK-activated plasmin cleaved the chromogenic substrate S-2251 (solid lines). Plasminogen was immobilized, SAK was added together with S-2251, and the cleavage of S-2251 was monitored at 405nm. The human plasminogen activator uPa was included as positive control (dashed lines). (C) SAK-activated plasmin degraded fibrinogen. SAK, plasminogen, and fibrinogen were incubated at 37°C, the protein mixture was separated by SDS-PAGE, and fibrinogen was detected using Western blot analysis. (A-C) A representative result out of two independent experiments is shown.

3.2.3 Plasmin or aureolysin degrades and inactivates SHL14

As apoE-derived SHL14 was shown to be a potent AMP, degradation of this AMP by host derived plasmin and staphylococcal aureolysin was investigated. Increasing amounts of purified plasmin (1:2080 to 1:208) or aureolysin (molar ratio enzyme to peptide 1:1000 to 1:50) were incubated with SHL14. The progression of the SHL14 degradation was visualized by separating the reaction mixtures by SDS-PAGE and staining with Coomassie. Both, plasmin and aureolysin, degraded SHL14 (FIG. 24 A-C). Plasmin-mediated SHL14 degradation was blocked by the serine protease inhibitor aprotinin (FIG. 24 A, lane 8). In addition to purified

plasmin, degradation of SHL14 by SAK-activated plasmin was analyzed. Again, complete degradation of the peptide was observed (FIG. 24 B, lane 6).

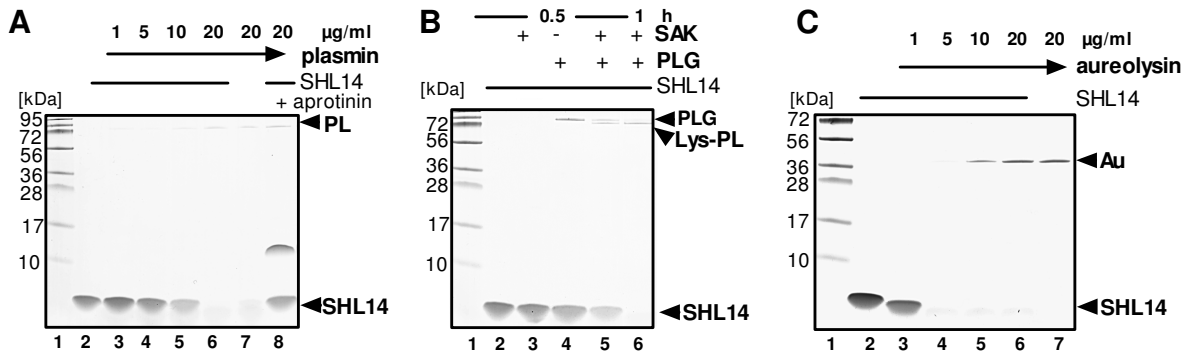

FIG. 24: Plasmin or aureolysin degrades SHL14.
(A) SHL14 was degraded by plasmin. SHL14 was mixed with the indicated amounts of plasmin (PL) and aprotinin. Samples were subjected to SDS-PAGE and stained with Coomassie. Plasmin-mediated proteolysis of SHL14 was inhibited by serine protease inhibitor aprotinin. (B) SHL14 was also degraded by SAK-activated plasmin. SHL14 was incubated with PLG and SAK for 0.5 or 1 h and the cleavage was assayed by SDS-PAGE and Coomassie staining. (C) SHL14 was degraded by aureolysin. SHL14 was incubated with increasing amounts of aureolysin (Au), separated by Tris-Tricine electrophoresis, and stained by Coomassie. (A-C) A representative result out of three independent experiments is shown.

Having demonstrated that plasmin and aureolysin degrade SHL14, the effect of supplemented aureolysin or plasmin on the interaction of $SHL14_{FITC}$ with Texas-Red-stained *S. aureus* was analyzed by fluorescent microscopy. $SHL14_{FITC}$ bound to bacteria (FIG. 25 A, panel I), but in the presence of plasmin or aureolysin $SHL14_{FITC}$ did not interact with *S. aureus* as shown by lack of green fluorescence (panel III or IV). By contrast, the protease HNE did not influence the interaction between SHL14 and the staphylococcal surface (panel II).

Finally, we examined the proteolytic effect of plasmin or aureolysin on SHL14 antibacterial activity using bacterial survival assays. *S. aureus* was incubated with increasing amounts of plasmin or aureolysin together with SHL14. Both proteases significantly reduced SHL14-mediated killing of the bacteria, whereas HNE did not influence the bactericidal activity of SHL14 (FIG. 25B).

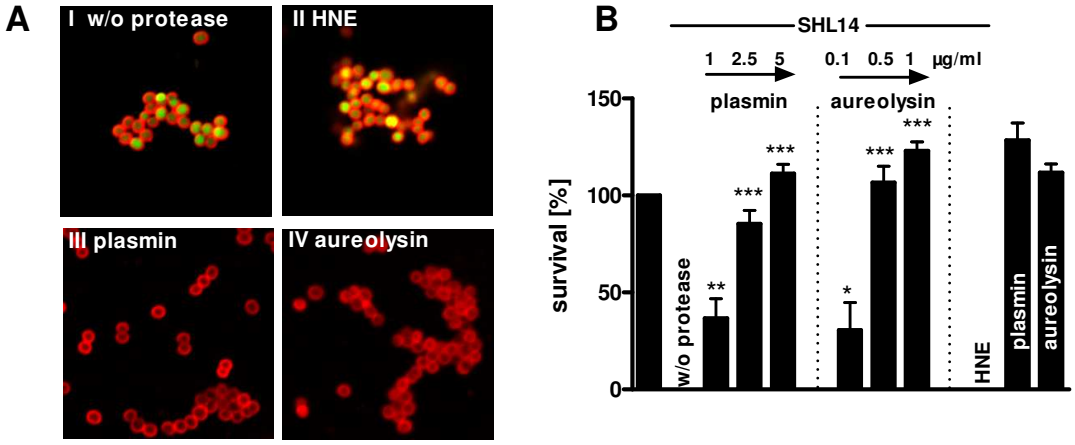

FIG. 25: Plasmin or aureolysin inactivates SHL14 activity.
(A) SHL14 binding to *S. aureus* was inhibited by plasmin or aureolysin. Texas-Red stained *S. aureus* was incubated with $SHL14_{FITC}$ in the presence of buffer (I), HNE (II), plasmin (III), or aureolysin (IV) and visualized by LSM. (B) SHL14 bactericidal activity was inhibited by plasmin or aureolysin. *S. aureus* was mixed with SHL14 and aureolysin, plasmin, or HNE and bacterial growth was followed by measuring OD_{650} after 9 h. HNE did not affect bactericidal activity of SHL14, thus no bacteria survived. Data shown are mean values of at least three independent experiments and error bars represent standard deviations. **p* <0.05; ***p* <0.01 and ****p* <0.001.

3.2.4 Plasmin or aureolysin degrades and inactivates apoE

After plasmin and aureolysin were shown to degrade and inactivate apoE-derived SHL14, the effect of these proteases was also assessed on full-length apoE. Plasmin (PLG+SAK) or aureolysin was incubated with apoE and proteolysis of apoE was assayed by SDS-PAGE and Coomassie staining. Plasmin degraded apoE into several fragments with mobilities ranging from 6 to 25 kDa (FIG. 26). By contrast, aureolysin showed a different cleavage pattern of apoE, as demonstrated by the appearance of two cleavage products with mobilities of 18 and 20 kDa.

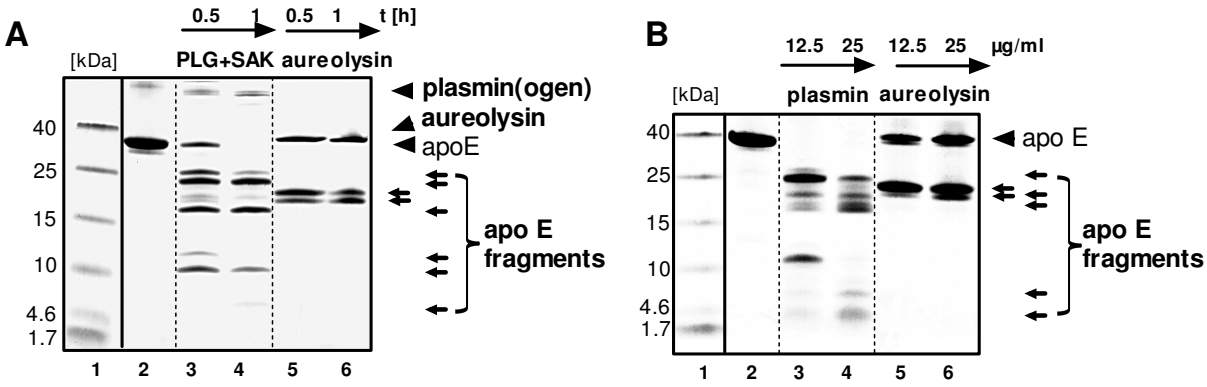

FIG. 26: Plasmin or aureolysin degrades apoE.
(A+B) ApoE was degraded by plasmin or aureolysin. (A) ApoE was incubated with plasmin (PLG+SAK) or aureolysin. Samples were taken after at 0.5 or 1 h, separated by SDS-PAGE, and visualized by Coomassie staining. (B) ApoE was incubated with the

indicated amounts of aureolysin or plasmin and analyzed as described for A. (A+B) A representative result out of three independent experiments is shown.

To determine whether proteolysis of apoE by plasmin or aureolysin affects apoE deposition on *S. aureus*, increasing concentrations of both proteases were added together with biotinylated apoE ($apoE_b$) to the bacteria. After washing, remaining surface-bound $apoE_b$ was analyzed by flow cytometry. Plasmin or aureolysin substantially decreased apoE deposition on *S. aureus* (FIG. 27 A).

The impact of the proteolytic activity of plasmin or aureolysin was then investigated on apoE-mediated adherence/phagocytosis. *S. aureus* was incubated with apoE and plasmin or aureolysin, stained with DID, added to DIO-labeled THP1 macrophages, then ingested and adsorbed bacteria were measured by flow cytometry Both proteases inhibited the phagocytosis-enhancing effect of apoE (FIG. 27 B). Thus, *S. aureus* uses plasmin and aureolysin to degrade apoE in order to evade adherence/phagocytosis caused by apoE. However, as plasmin and aureolysin decreased adherence/phagocytosis below the basal level (dotted line) an additional unknown activity of these proteases on phagocytosis is anticipated.

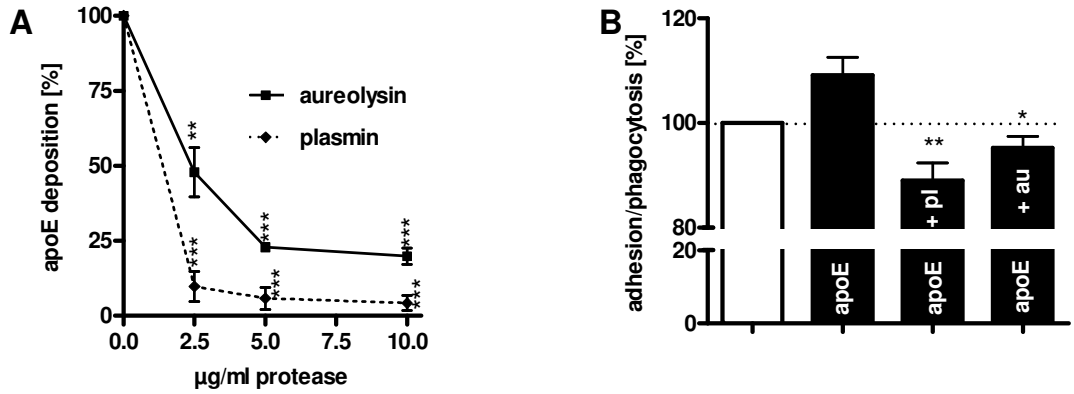

FIG. 27: Plasmin or aureolysin inactivates apoE activity.

(A) ApoE deposition on the staphylococcal surface was inhibited by plasmin or aureolysin. Bacteria were mixed with biotin-labeled apoE and aureolysin or plasmin. After extensive washing, surface deposited apoE was determined by flow cytometry. ApoE binding to *S. aureus* (without protease) was set to 100%. (B) ApoE-mediated adhesion/phagocytosis is inhibited by plasmin or aureolysin. Phagocytosis assays were performed as described before and aureolysin or plasmin was added to apoE treated bacteria. Adhesion/ phagocytosis without apoE was set to 100%. (A+B) Data represent mean values ± standard deviations of three independent experiments; *p <0.05; **p <0.01 and ***p <0.001.

3.3 Plasmin & Sbi or Efb act in concert during staphylococcal complement evasion

Plasmin is also exploited by *S. aureus* to counteract complement attack by degrading C3b (116). In the following chapter the plasmin-mediated cleavage of C3, C3b, and C3a were investigated. In addition, the impact of the staphylococcal C3- and C3b-binding proteins Sbi and Efb on plasmin-mediated cleavage of C3 and C3b was characterized.

3.3.1 Plasminogen is recruited by Sbi or Efb

First of all, it was investigated whether the C3-binding proteins, Sbi and Efb, bind to plasminogen. Biotin-labeled plasminogen (PLG_b) was incubated with immobilized Sbi or Efb and assayed for binding. Plasminogen bound to both staphylococcal proteins. The accuracy of recombinant Sbi and Efb was confirmed as both bound to C3 and Sbi bound to IgG. Plasminogen binding to Sbi and Efb was similar to the previously identified plasminogen ligand CRASP-5 of *Borrelia burgdorferi* (130) (FIG. 28 A+B). To exclude the possibility of nonspecific binding of these staphylococcal proteins to the biotin-label of plasminogen, binding of also unlabeled plasminogen to Sbi or Efb was tested using a combined ELISA-Western blot assay (CEWA). Plasminogen was added to immobilized Sbi or Efb, washed, and all surface bound proteins were separated by SDS and immunoblotted for the detection of plasminogen. Free plasminogen, similar to PLG_b, bound to Sbi, Efb, and to CRASP-5 (FIG. 28 C).

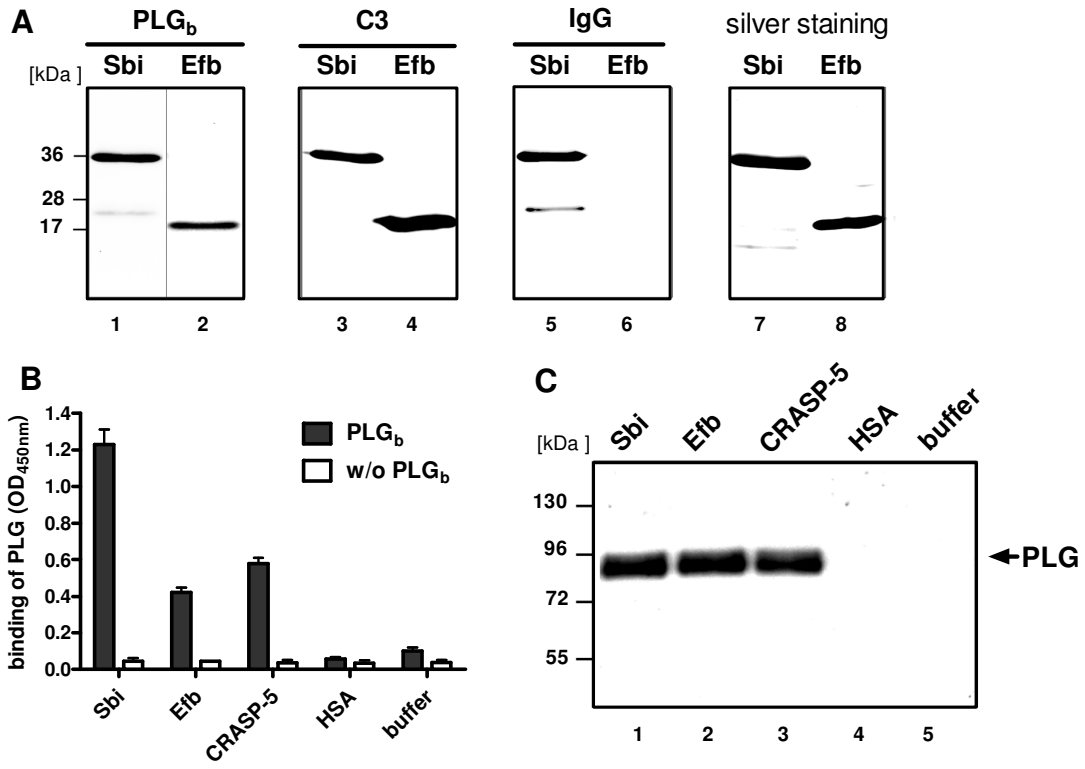

FIG. 28: Plasminogen is recruited by Sbi and Efb.
(A) Plasminogen bound to Sbi and Efb (lane 1 and 2). The accuracy of the recombinant staphylococcal proteins was confirmed; as both, Sbi and Efb, bound to C3 (lane 3 and 4), but only Sbi bound IgG (lane 5 and 6). Sbi or Efb was separated by SDS-PAGE, and either blotted to a membrane (lane 1-6) or stained with silver nitrate (lane 7-8). Membranes were incubated with biotinylated PLG (PLG$_b$), C3, or HRP-coupled Ab and bound proteins were detected as indicated. (B) Binding of plasminogen to Sbi or Efb was confirmed by ELISA. Bacterial proteins (equimolar) were immobilized; PLG$_b$ was applied, and detected using streptavidin-HRP. Plasminogen binding to Sbi and Efb was comparable to plasminogen binding of the borrelial protein CRASP-5. Plasminogen did not bind to HSA or to the plate (buffer). Data represent mean values and standard deviations of three independent experiments. (C) Plasminogen was recruited by Sbi and Efb. Nonlabeled plasminogen was added to immobilized bacterial proteins. Bound plasminogen was eluted, separated by SDS-PAGE, and detected by Western blot analysis. Plasminogen was recruited to CRASP-5, and no unspecific binding to HSA or the plate (buffer) was detected. A representative result out of three independent experiments is shown.

3.3.2 Characterization of the plasminogen:Sbi/Efb interactions

To determine the Sbi and Efb domains responsible for interaction with plasminogen, plasminogen binding to the structurally related domains Sbi 3-4 and Efb-C, in Sbi and Efb, respectively, was assayed. Plasminogen, added to the immobilized domain fragments, bound to both Sbi 3-4 (FIG. 29 A) and Efb-C (FIG. 29 B) in a dose-dependent manner. These binding interactions were also followed in real time using surface plasmon resonance. Plasminogen added in fluid phase bound to immobilized Sbi 3-4 or Efb-C and confirmed the interaction between

plasminogen and these specific Sbi- and Efb-domains. To investigate whether plasminogen binding to Sbi and Efb is similar to human proteins, which bind plasminogen via lysine residues, the lysine analog εACA, was used in binding assays. εACA interfered with plasminogen binding to both, Sbi 3-4 and Efb-C (FIG. 29 B+C), demonstrating that lysine residues are involved in the interactions with plasminogen.

We next asked whether plasminogen and C3 can bind simultaneously to Sbi and Efb. Therefore, increasing amounts of plasminogen were added together with constant amounts of C3 to immobilized Sbi 3-4 or Efb-C and plasminogen and C3 binding were assayed. C3 binding was not reduced when plasminogen binding increased. These results indicate that plasminogen does not interfere with the C3 binding to Sbi 3-4 or Efb-C (FIG. 29 E or F) and that plasminogen and C3 bind simultaneously to both staphylococcal molecules**.**

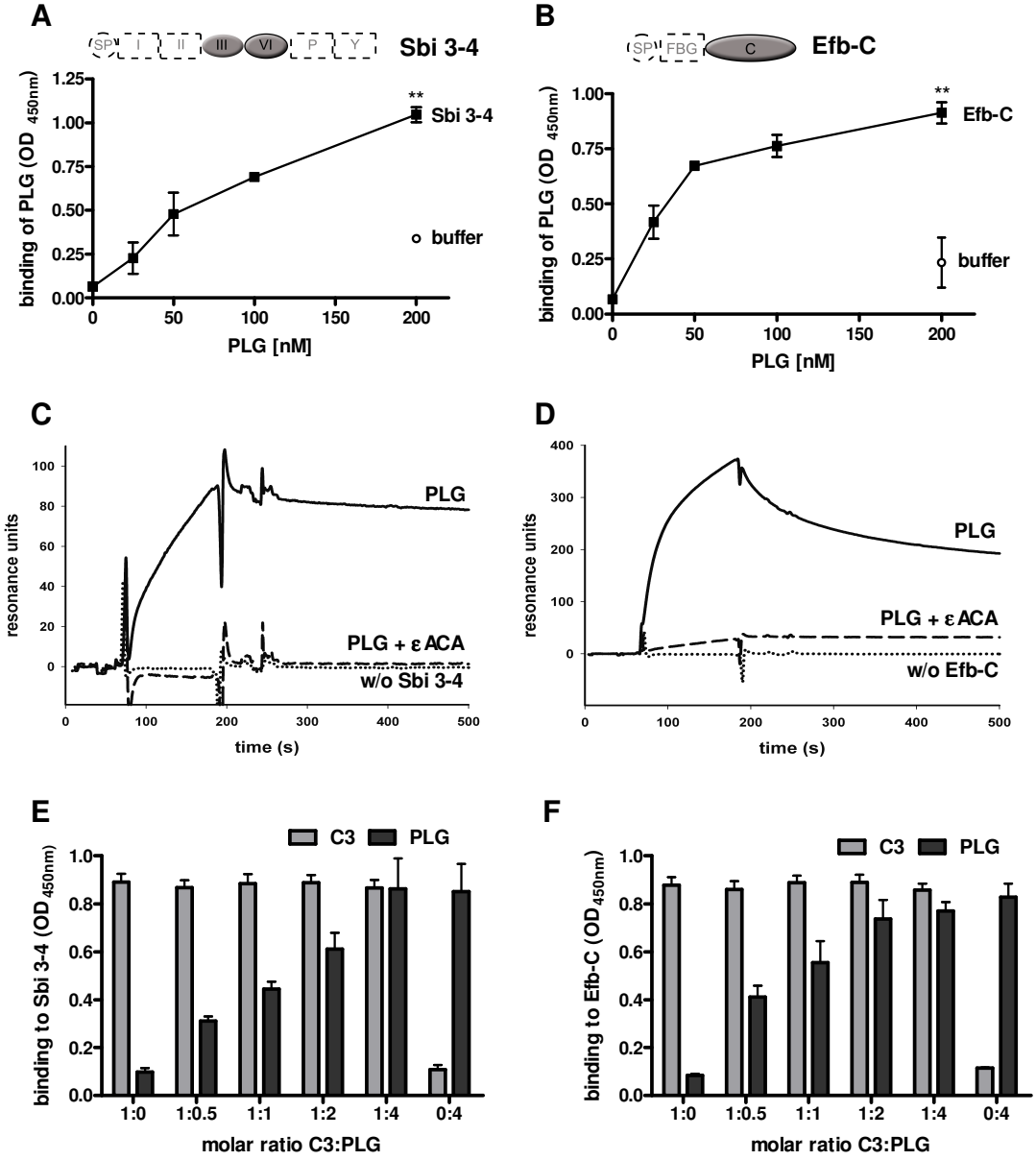

FIG. 29: Characterization of the plasminogen interactions with Sbi 3-4 and Efb-C.
(A) Plasminogen (PLG) bound to the fragments 3-4 of Sbi and (B) the C-terminus of Efb. Sbi 3-4 or Efb-C was immobilized and increasing amounts of plasminogen were added. Bound plasminogen was detected with specific antisera. (C) Plasminogen (solid lines) associated with Sbi 3-4 or (D) Efb-C determined by surface plasmon resonance. Sbi 3-4 or Efb-C was immobilized, and plasminogen was applied in fluid phase. Binding of plasminogen to Sbi 3-4 or Efb-C was inhibited by εACA (dashed line). A representative experiment out of three is shown. (E) Plasminogen (black columns) and C3 (grey columns) bound simultaneously to Sbi 3-4 and (F) Efb-C. Sbi 3-4 or Efb-C was immobilized, and constant amounts of C3 together with increasing concentrations of plasminogen were added. (A, B, E, F) Data represent mean values and standard deviations of three independent experiments. *p <0.05 and **p <0.01.

3.3.3 Plasminogen bound to Sbi or Efb is activated to plasmin

To exert proteolytic activity, plasminogen is converted to plasmin by interaction with a human or bacterial activator. Therefore, activator accessibility of Sbi- or Efb-

bound plasminogen was investigated. Plasminogen was immobilized with Sbi or Efb, treated with the human activator uPa, and the subsequent cleavage of a plasmin-specific chromogenic substrate was monitored. Sbi- or Efb-bound plasminogen was activated by uPa to plasmin, as demonstrated by the increased conversion of the substrate S-2251. Similarly, CRASP-5-bound plasminogen was activated to the protease plasmin (FIG. 30).

SAK is known to activate plasminogen via a nonproteolytic mechanism. Experiments were performed to determine whether SAK is also able to activate Sbi- or Efb-bound plasminogen. SAK and human uPa were shown to activate plasminogen bound to Sbi or Efb and the resulting plasmin cleaved the chromogenic substrate (FIG. 30 B or C). In summary, plasminogen bound to Sbi and Efb is activated by both, staphylococcal and human activators, to form active plasmin.

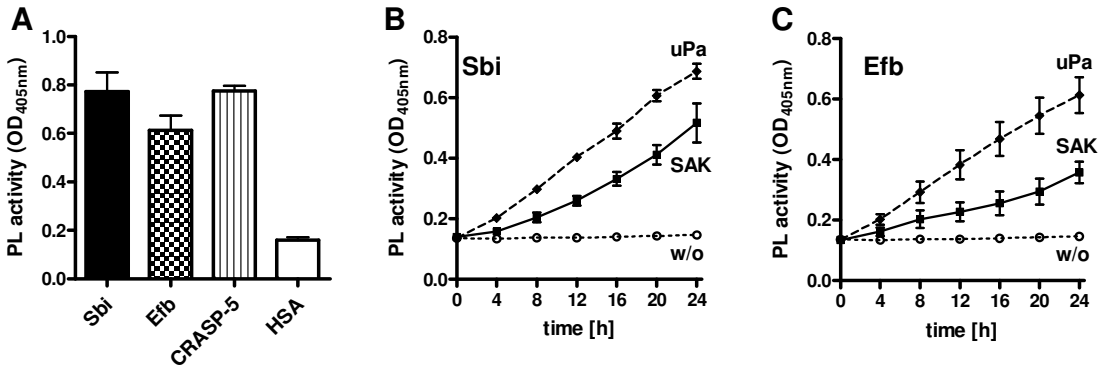

FIG. 30: Plasminogen bound to Sbi or Efb is converted to plasmin.
(A) Plasmin bound to Sbi, Efb, and the borrelial CRASP-5 converted a chromogenic substrate, as monitored by measuring absorbance at 405 nm. Equimolar amounts of bacterial proteins were immobilized and incubated with plasminogen. After washing, the activator uPa was applied together with the chromogenic substrate S-2251. Plasminogen did not bind to human serum albumin (HSA), thus no plasmin activation was observed. (B) Plasminogen bound to Sbi and (C) Efb was activated by uPa or SAK to plasmin. Plasmin activators uPa or SAK were applied to plasminogen bound to Sbi or Efb together with the chromogenic substrate. Conversion of S-2251 was assessed at various time points. Data represent mean values and standard deviations of three independent experiments.

3.3.4 Characterization of the plasmin-mediated C3 degradation

To analyze the impact of Sbi and Efb on plasmin-mediated C3 degradation, at first C3 degradation by plasmin was analyzed. C3 and its fragments C3b, iC3b, and C3c were incubated with plasmin and the resulting cleavage patterns were visualized by SDS-PAGE and Western blotting using anti-C3 Ab. Plasmin degraded all C3 fragments, leading to similar cleavage products at 114 (α´), 68, 40, 27, 20, and 17 kDa (FIG. 31 A). These C3 products indicate that during

plasmin-mediated C3 cleavage C3b (114 kDa), iC3b (68 kDa), and C3c (40 and 27 kDa) intermediates are generated. Plasmin also degraded the β-chain of C3 as demonstrated by the reduced amount of the β-chain in lane 6 and 8.

Additionally, plasmin cleavage was compared to factor I-mediated cleavage of C3 and its fragments by performing SDS-PAGE and Western blotting using anti-C3 Ab. Factor I and its cofactor factor H degraded C3 and C3b, but not iC3b (FIG. 31 B). C3 degradation by factor I is presumable caused by C3(H_20) instead of C3 in the reaction. The addition of both proteases and factor H resulted in enhanced cleavage of C3 and C3b (lane 4 and 8), as less C3 α- and α´-chains were detectable. Because iC3b was not cleaved by factor I and factor H, no enhancing effect was observed for iC3b cleavage. These results for C3 degradation by plasmin were summarized in a schematic overview modified after Nagasawa et *al.* and Mizuno et *al.* (53, 131) (FIG. 31 C).

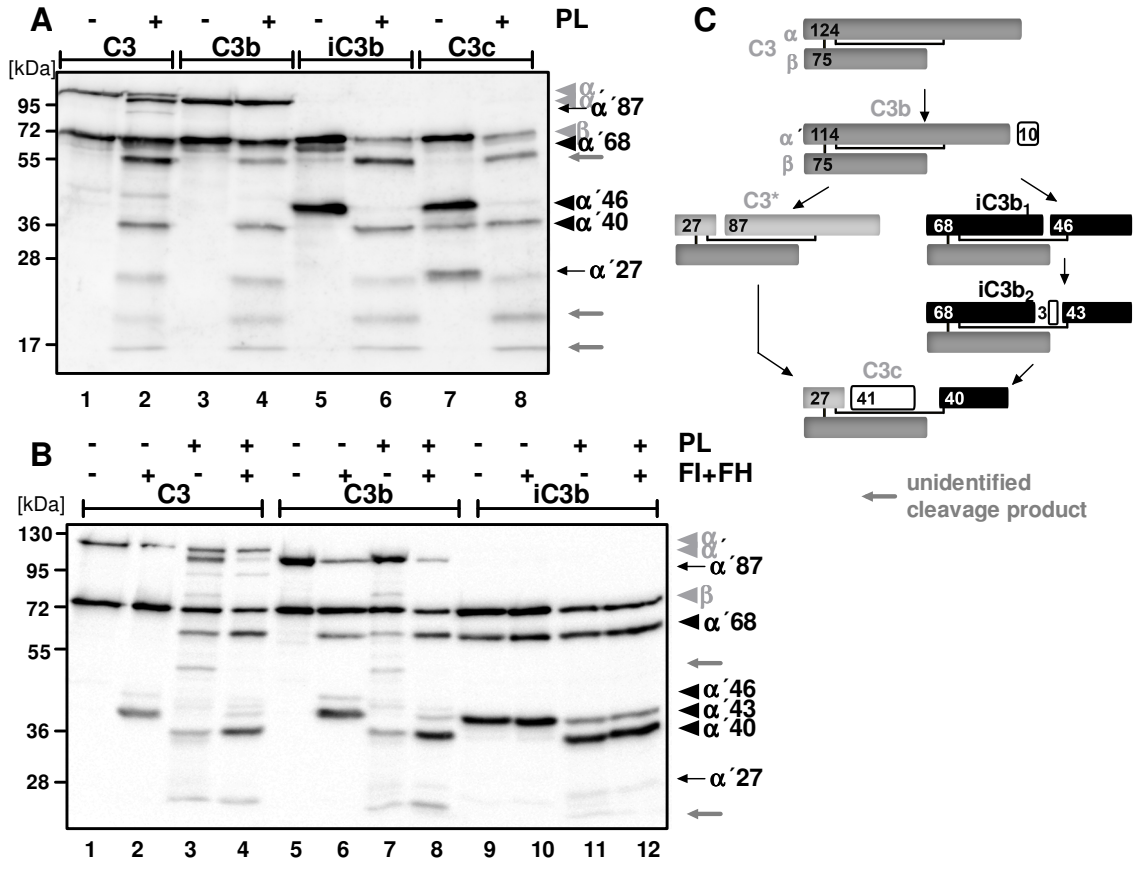

FIG. 31: Characterization of plasmin-mediated cleavage of C3 and C3 fragments.
(A) Plasmin degraded C3, C3b, iC3b, and C3c. Plasmin was incubated with C3 and C3 fragments; samples were separated by SDS-PAGE and Western blotted using anti-C3 pAb. (B) Plasmin- and factor I-mediated cleavage of C3, C3b, and iC3b. Plasmin or/and factor I (FI) plus factor H (FH) were incubated with C3 fragments. Samples were analyzed as described for (A). (C) Schema of the C3 cleavage products generated by plasmin. All products in white boxes (C3a, C3f, C3dg) are not recognized by anti-C3 pAb or anti-C3-HRP.

3.3.5 Plasmin bound to Sbi or Efb degrades C3 and C3b

After plasmin(ogen) was shown to form complexes with C3 and Sbi or Efb, the proteolytic plasmin activity against complexed C3 was assayed. Plasminogen was bound together with C3 to immobilized Sbi or Efb and activated by the addition of SAK. The progression of C3 degradation was followed by separating the reaction mixture by SDS-PAGE and Western blot analysis using anti-C3 Fab´-fragments. Sbi- or Efb-bound plasminogen was activated by SAK to plasmin which subsequently cleaved bound C3, as shown by multiple C3-degradation products with mobilities of 114, 87, 68, 40, and 27 kDa (FIG. 32 A).

In a similar assay performed with C3b, plasmin was observed to also degrade complexed C3b (FIG. 32 B), as demonstrated by the appearance of cleavage products with mobilities of 87, 68, 40, and 27 kDa. When CRASP-5 or HSA was used in these assays, instead of Sbi or Efb, no C3 cleavage was observed. Thus, plasmin degrades the complement proteins C3 and C3b when complexed with Sbi or Efb.

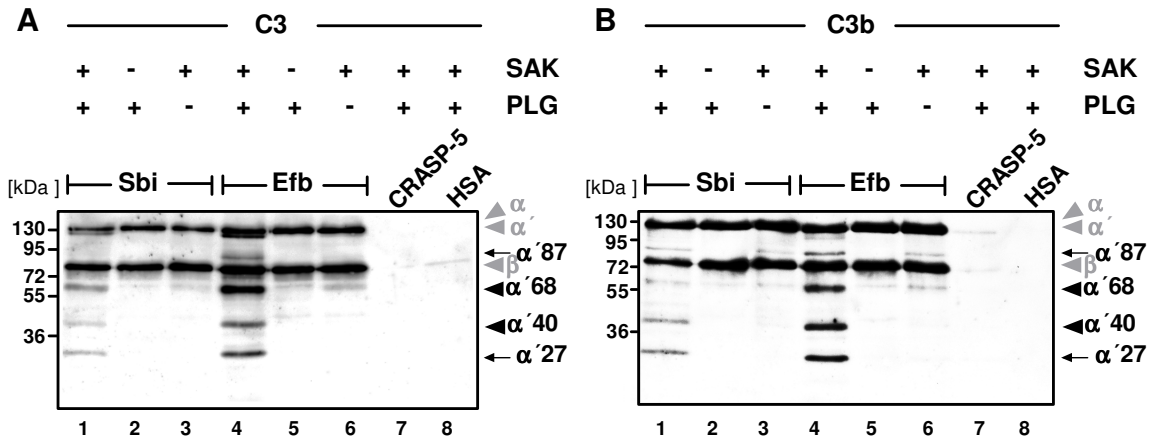

FIG. 32: Plasmin complexed with C3/C3b and Sbi or Efb degrades C3 and C3b within the complex.

(A) Plasmin degraded C3 or (B) C3b within the Sbi:plasmin:C3/C3b or Efb:plasmin:C3/C3b complexes (lane 1 and 4), and C3/C3b cleavage products (marked with arrows) appeared. Bacterial proteins were immobilized (equimolar) and plasminogen was added together with C3 or C3b. SAK was applied for 3 h and C3/C3b cleavage was analyzed by Western blot analysis using anti-C3-HRP (Fab). The α-/α´- and β-chains of C3/C3b and the cleavage products are indicated by arrows. For CRASP-5 and HSA no C3 cleavage products were observed (lane 7 and 8). Data shown are representative of three independent experiments.

3.3.6 C3 degradation by plasmin is enhanced by Sbi and Efb

Upon binding, Efb changes the structural conformation of C3, leading to an increased susceptibility of C3 to degradation by trypsin (100). To analyze whether

C3 degradation by plasmin is also modulated by Sbi or Efb, C3 proteolysis by plasmin was compared in the presence or absence of Sbi or Efb. C3 degradation by plasmin was clearly enhanced by both staphylococcal proteins, as demonstrated by the appearance of additional C3 cleavage products in Western blot analysis using anti-C3 Fab´-fragments for protein detection (FIG. 33). By contrast, proteolytic cleavage of C3 by plasmin was not affected after addition of CRASP5 or HSA.

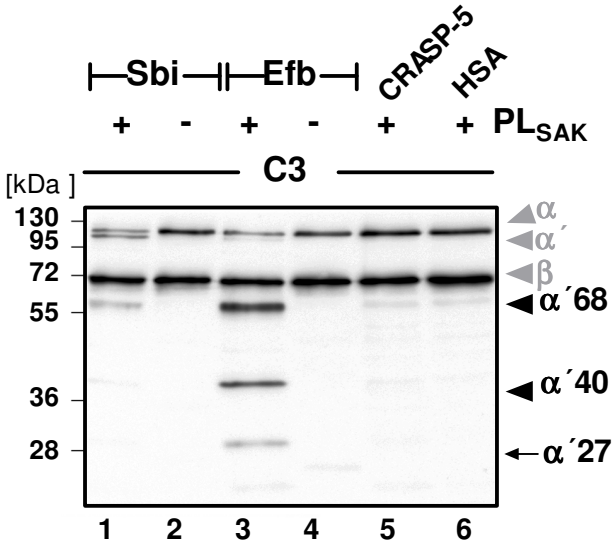

FIG. 33: Plasmin-mediated cleavage of C3 is enhanced by Sbi and Efb. Sbi or Efb (lane 1 or 3) enhanced plasmin-mediated degradation of C3, as demonstrated by the decrease of the α-chain, and the increase of C3 cleavage products (indicated by arrows). CRASP-5 showed no enhancing-degradative effect. C3 was incubated with PL_{SAK} (PLG+SAK) in the presence of Sbi, Efb, CRASP-5, or HSA. Samples were separated by SDS-PAGE, and C3 cleavage products were visualized by Western blot analysis using anti-C3-HRP (Fab).

3.3.7 Cloning, expression, and purification of Sbi 1-2

To specify the impact Sbi or Efb exerts on plasmin-mediated C3 degradation, the non C3-binding Sbi construct Sbi 1-2 was cloned and expressed. This construct comprises the two N-terminal IgG-binding domains and lacks the signal sequence (FIG. 34 A). A blunt-end PCR product was generated, with a motility of 343 bp (FIG. 34 B), inserted into the pET101 vector, and transformed in *E. coli* Top10 cells. Six of the resultant colonies were screened for the presence of the *sbi 1-2* construct. Two plasmids that contained an inserts of 343 bp, were isolated and sequenced (FIG. 34 C). Sequence analysis showed that the *sbi 1-2* gene was correctly inserted into the expression vector. Plasmids were then used to transform the *E. coli* expression strain BL21 Star, and protein expression was induced by addition of IPTG. Sbi 1-2 was purified according to section 2.7. Purified protein was visualized with SDS-PAGE and Western blotting or silver staining, and showed the mobility of the predicted molecular mass of ~16 kDa (FIG. 34 D).

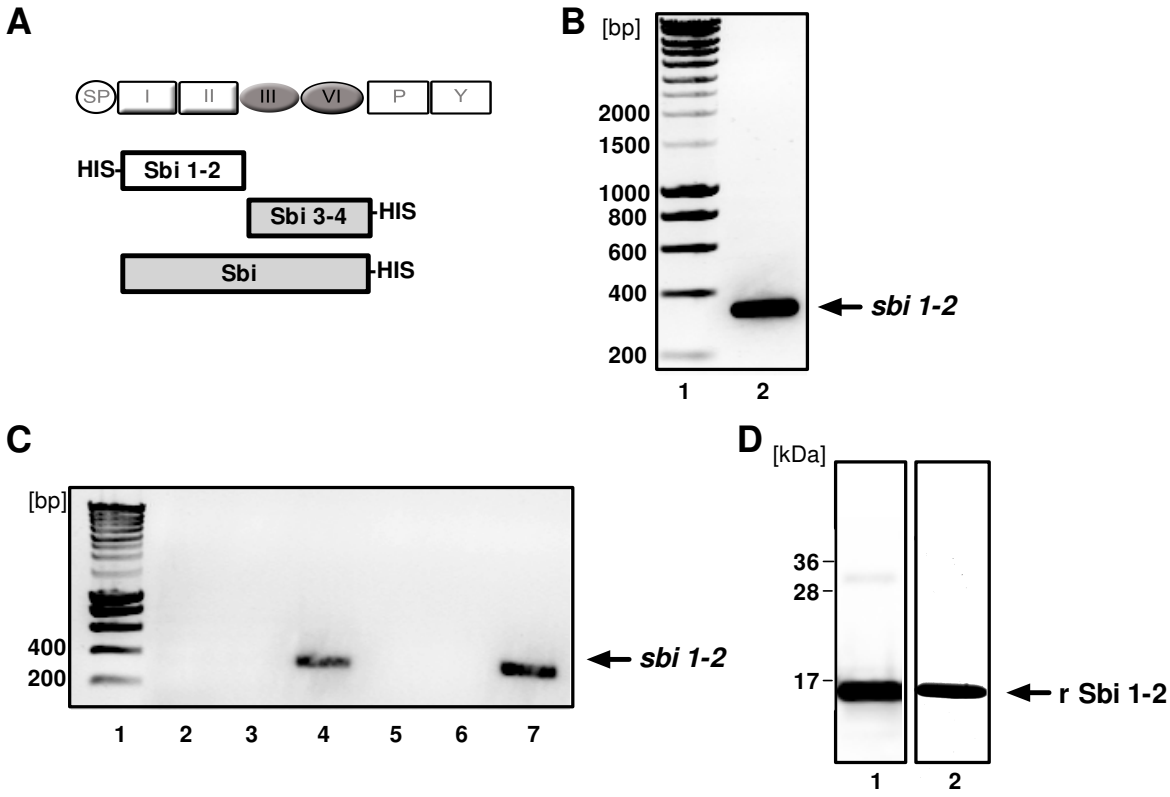

FIG. 34: Cloning, purification, and expression of Sbi 1-2.
(A) Schema of the Sbi constructs used in this thesis. Sbi 1-2, which encompasses the first two IgG-binding domains, was clones and expressed. Sbi 1-4 (in the following referred as Sbi) and Sbi 3-4 were cloned previously. (B) The *sbi 1-2* construct was amplified by PCR and yielded a 343 bp blunt-end product. (C) PCR screening for pET101 *sbi 1-2* positive clones. The PCR product was inserted in pET101, and transformed into *E. coli* Top10 cells. Six clones were tested by colony PCR (lanes 2 to 7) and two positive clones were indentified (lane 4 and 7). (D) Recombinant Sbi 1-2 migrated at ~16 kDa (arrow). Purified Sbi 1-2 was separated by SDS-PAGE and visualized by immunoblotting (lane 1) using anti-goat-HRP (using the IgG-binding property of the Sbi 1-2) or stained by silver nitrate (lane 2).

3.3.8 C3 degradation by plasmin is enhanced by Sbi 3-4 and Efb-C

To localize the Sbi and Efb domains responsible for the enhancement in C3 degradation by plasmin, the effect of Sbi 1-2; Sbi 3-4 and Efb-C were investigated. C3 cleavage by plasmin was enhanced in the presence of Sbi 3-4 and Efb-C, but not in the presence of Sbi 1-2 (FIG. 35 A). To confirm these results, C3 proteolysis was also examined using ELISA. C3 was immobilized on a microtiter plate and plasmin (converted by uPa or SAK) was added together with the bacterial proteins or with HSA. After incubation, the plates were washed, and the amount of bound C3 was determined. Smaller amounts of C3 were detected in reactions containing plasmin than on those containing plasminogen. The plasmin-dependent effect on C3 deposition was enhanced by the addition of Sbi 3-4 and especially Efb-C to the reaction mixture, but not by the addition of CRASP5 or HSA (FIG. 35 B). This

enhancement by Sbi 3-4 and Efb-C was more clearly demonstrated by using an assay in which increasing amounts of the bacterial proteins were used (FIG. 35 D). The results indicate that plasmin cleaves C3, and that the staphylococcal proteins Sbi or Efb enhance the cleavage by plasmin. The domains responsible for the degradation-enhancing effect were located to the domains Sbi 3-4 and Efb-C, each of which contains a three-helix bundle motif.

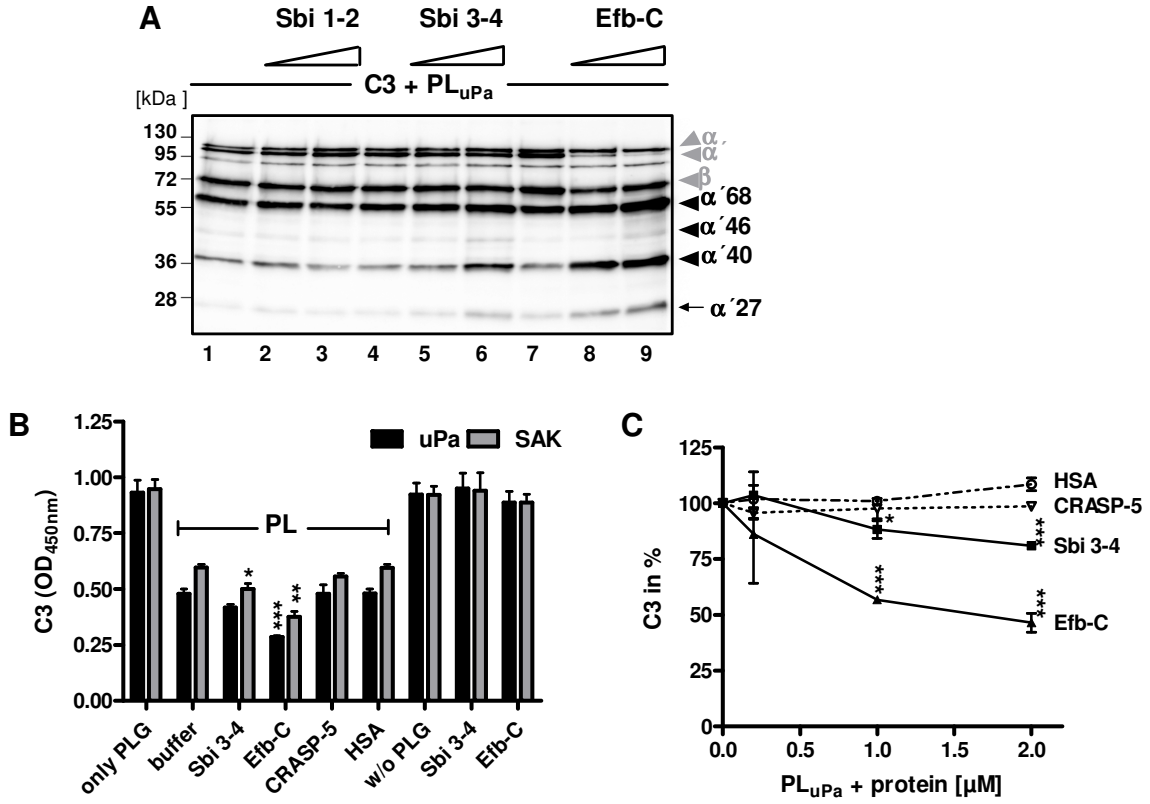

FIG. 35: Plasmin-mediated cleavage of C3 is enhanced by Sbi 3-4 and Efb-C.
(A). Sbi 3-4 (lane 5 and 6) and Efb-C (lane 8 and 9) enhanced C3 cleavage, as shown by the increase in C3 cleavage products in Western blot analysis using anti-C3 pAb. The IgG-binding Sbi 1-2 (mobility of 16 kDa) did not affect the C3 cleavage pattern (lane 2 and 3). (B) The degradation-enhancing effect of Sbi 3-4 and Efb-C was confirmed by ELISA. C3 was immobilized and plasmin (PLG+uPa or PLG+SAK) together with Sbi 3-4 or Efb-C were added. C3 deposition was detected after 3 h with anti-C3a Ab. CRASP-5 and HSA did not influence C3 cleavage. (C) Sbi 3-4 and Efb-C enhanced C3 degradation by plasmin in a dose dependent manner. C3 incubated only with plasmin was set to 100%. Data in B and C represent mean values of three independent experiments and error bars represent standard deviations.*p <0.05, **p <0.01, and ***p <0.001.

3.3.9 C3b degradation by plasmin is also enhanced by Sbi 1-2 and Efb-C

Having shown that plasmin cleaves C3 more efficiently in the presence of Sbi or Efb, the same effect was analyzed for C3b. Plasmin-mediated C3b degradation was assayed using again deletion fragments Sbi 1-2, Sbi 3-4 or Efb-C, in the same assay as described for C3. The C3b degradation by plasmin was enhanced in the presence of Sbi 3-4 or Efb-C, as shown by the increase in C3b degradation

products (FIG. 36 A). However, the addition of Sbi 1-2 to C3b resulted in no enhancement effect. To investigate whether Sbi and Efb also accelerate plasmin activity on the bacterial surface, *S. aureus* was incubated with human serum to allow complement-mediated C3b deposition. The C3b-coated bacteria were then washed, incubated with plasmin plus Sbi 3-4 or Efb-C, and C3b deposition was analyzed by flow cytometry. Plasmin substantially reduced the C3b opsonization of *S. aureus* by about 37%. In the presence of Sbi 3-4, C3b deposition was further decreased to 50% and in the presence of Efb-C to 38% (FIG. 36 B). In parallel analyses, supernatants containing the C3b degradation products were characterized by SDS-PAGE and Western blotting. C3b cleavage products with mobilities of 41 and 27 kDa were identified in the supernatants of those samples containing C3b-opsonized *S. aureus* treated with plasmin (FIG. 36 C). Again C3b degradation was accelerated when plasmin cleaved surface-bound C3b in the presence of Sbi 3-4 and Efb-C (indicated by arrows). By contrast, the presence of Sbi 1-2 showed no effect. These results demonstrate that C3b degradation by plasmin is accelerated by interaction with the staphylococcal proteins Sbi and Efb on the bacterial surface.

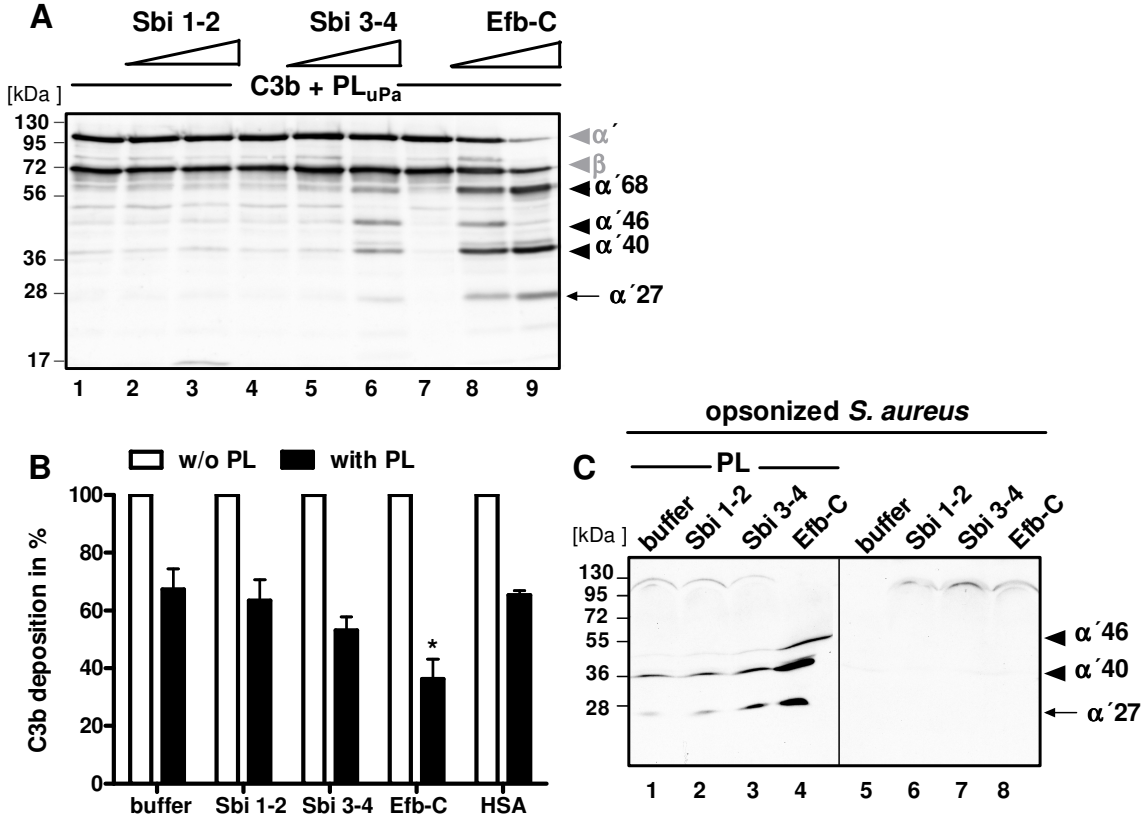

FIG. 36: Plasmin-cleavage of C3b is enhanced by Sbi 3-4 and Efb-C.
(A) Sbi 3-4 (lane 5-6) and Efb-C (lane 8-9) enhanced plasmin-mediated cleavage of C3b, as demonstrated by the accumulation of C3b cleavage products. Sbi 1-2 did not influence

C3b degradation (lane 2-3). (B+C) Sbi 3-4 and Efb-C enhanced the anti-opsonic activity of plasmin. C3b was deposited on *S. aureus;* bacteria were then incubated with Sbi 1-2, Sbi 3-4, Efb-C or HSA in the presence or absence of plasmin. Remaining C3b on *S. aureus* was measured by flow-cytometry The C3b generation in the absences of plasmin was set to 100%. Data represent mean values and standard deviations of five independent experiments.*p <0.05. (C) In parallel the supernatants were separated by SDS-PAGE and analyzed by Western blotting using anti-C3-HRP (Fab). Sbi 3-4 (lane 2) and Efb-C (lane 4) increased the plasmin degradation, as shown by the increase in C3b cleavage products. However, Sbi 1-2 did not influence the amount of C3b-fragments (lane 1). (A+C) A representative result out of three independent experiments is shown.

3.3.10 Further characterization of the degradation-enhancing effect

After Sbi 3-4 and Efb-C were identified to enhance plasmin-mediated degradation of C3 and C3b, we assessed the effect for the iC3b fragment in the previously described assay. Neither Sbi 3-4 nor Efb-C increased the susceptibility of iC3b to degradation by plasmin (FIG. 37 A). Thus, the degradation-enhancing activity of the staphylococcal protein was restricted to C3 and C3b.

To further characterize this effect, C3 and C3b degradation by an additional protease were assayed in the presence of Efb-C. Aureolysin was incubated with C3 or C3b in the presence or absence of Efb-C and plasmin was included as control. Samples were analyzed as described before. Efb-C also enhanced aureolysin-mediated degradation of C3 and C3b (FIG. 37 B), indicating that the degradation-enhancing Efb-C (or Sbi 3-4) also affect C3 and C3b proteolysis by additional proteases. However, in contrast to the aureolysin-mediated C3 cleavage pattern described by Laarman *et al.* (113), additional C3 cleavage products were observed with mobilities of 40 and 27 kDa (FIG. 37 C+D).

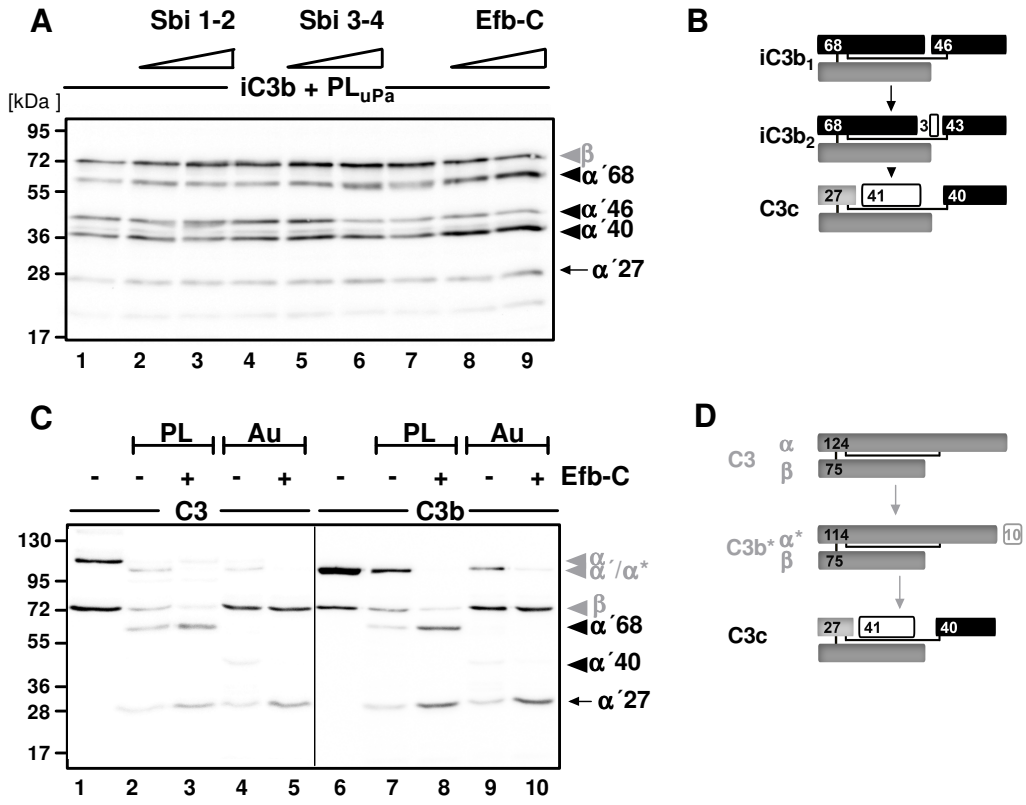

FIG. 37: Characterization of the degradation-enhancing effects of Sbi 3-4 and Efb-C. (A) Plasmin-mediated iC3b proteolysis is not affected by Sbi 1-2, Sbi 3-4, or Efb-C. Plasmin-mediated iC3b degradation was assayed as described for C3 or C3b, but iC3b was used instead of C3 or C3b. (B) Schematic overview over the plasmin-mediated cleavage of iC3b. (C) Efb-C enhances aureolysin-mediated degradation of C3 and C3b. C3 and C3b degradation assays were performed as described before, but besides plasmin (PL) also aureolysin (Au) was tested. (D) Schematic cleavage of C3 by aureolysin.

3.3.11 Plasmin degrades C3a

Plasmin cleaves C3 into functionally active C3a (52, 132) and proteolytically inactivates the opsonin C3b (51, 54, 116). Because C3a exhibits antimicrobial activity (32, 33) and thus kills *S. aureus,* we investigated whether *S. aureus* evades this antimicrobial attack by using plasmin to degrade also C3a. *S. aureus* was treated with C3a alone or with C3a together with plasmin (PL_{SAK}). The supernatants were analyzed by SDS-PAGE and Western blotting using anti-C3a Ab. Plasmin completely degraded C3a, as demonstrated by the absence of detectable levels of C3a (FIG. 38 A). When plasminogen was added without SAK to *S. aureus*, the amount of C3a was decreased by about 15%, which may be explained by synthesis and secretion of SAK by *S. aureus* during the assay. In parallel, C3a antimicrobial activity against *S. aureus* was analyzed in survival assays. C3a added to growing *S. aureus* killed the bacteria, but in the presence of C3a and plasmin (PLG+SAK) 88% of *S. aureus* survived. Plasminogen added

without SAK resulted in 50% survival of the bacteria, and addition of SAK without plasminogen had no effect on the bacteria (FIG. 38 B). Thus, plasmin inhibits the bactericidal activity of C3a by degradation of the C3a molecule.

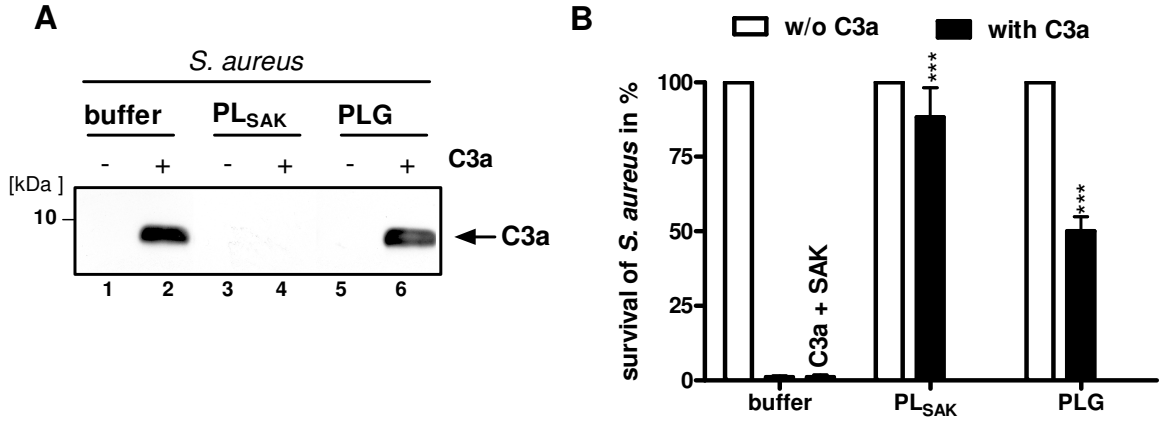

FIG. 38: Plasmin degrades and inactivates C3a.

(A) C3a was completely degraded by plasmin; plasminogen had a weak degrading effect on C3a. *S. aureus* was incubated with C3a in the absence or presence of plasmin (PLG+SAK) (lane 4) or plasminogen (lane 6). The supernatants were analyzed by SDS-PAGE and Western blotting. (B) Plasmin (PLG+SAK) inhibited C3a antibacterial activity, and increased the survival of *S. aureus* by 88% and plasminogen to 50%. *S. aureus* treated with C3a in the absence or presence of plasmin or plasminogen was cultivated overnight on LB agar plates. CFUs were counted and the survival without C3a was set to 100% (white columns). Data represent mean values ± standard deviations of three independent experiments. ***p<0.001.

4 DISCUSSION

Infections with pathogens usually result in the activation of the host immune response. The immune system recognizes pathogenic microbes and triggers a number of reactions counteracting the spreading of the invaders. Complement and coagulation are immediately initiated leading to (i) opsonization of the microbial intruders, (ii) generation of AMPs, (iii) release of chemotactic C3a and C5a, which recruit neutrophils and macrophages to the site of infection, (vi) killing of bacteria by TCC, and (v) entrapment of pathogens by fibrin clots. However, pathogenic microorganisms, such as *S. aureus,* have developed sophisticated strategies to counteract these host immune defense mechanisms.

In this study, the roles of apoE and the serine protease plasmin in the host-pathogen interactions were investigated. ApoE exerted bactericidal activity against *S. aureus* after HNE-mediated proteolysis and the intact protein was shown to target *S. aureus* and facilitate phagocytosis. Both functions of apoE were negatively regulated by plasmin. In addition, plasmin also inactivates a variety of complement components including C3, C3a, and C3b. Consequently, *S. aureus* hijacks plasminogen and subsequently activates plasmin to evade the apoE-mediated innate immune response and complement activity.

In summary, the human plasma proteins apoE and plasmin(ogen) play distinguished roles in the host-pathogen interactions. ApoE is part of the innate immune response, and is therefore proteolytically inactivated by *S. aureus*. By contrast, the activated plasma protease plasmin terminates innate immune reactions and is exploited by *S. aureus*.

4.1 Two novel immunomodulatory functions of apoE

Because apoE knock-out mice are susceptible to bacterial infections, apoE is implicated in immunity (64). Here, we characterized two novel immune functions of apoE. The protein exerted antibacterial activity against Gram-positive and -negative bacteria after cleavage by the neutrophil protease HNE and binds to *S. aureus* and facilitates phagocytosis.

4.1.1 ApoE-derived AMPs are generated by HNE

Although apoE consists of several amphipathic α-helixes, a common structural feature of AMPs (18), the intact apoE molecule exhibited no bactericidal activity.

When neutrophils are attracted to the site of infection, they release a variety of proteases, such as HNE (125, 126). ApoE was cleaved by proteases of the PMN lysate, as well as by purified HNE, into antimicrobial active fragments that kill *E. coli* and *S. aureus*. Hence, the antimicrobial sequence of apoE is hidden in the intact molecule and exposed after proteolytic processing. This observation concurs with previous reports showing that cleavage products of other plasma proteins including complement C3 (32, 33), HMWK (9), and β2-glycoprotein I (10) exert antimicrobial activities after proteolysis of the intact proteins. In addition, HNE itself harbors antimicrobial activity (125) and therefore the protease and plasma-derived AMPs likely act synergistically in killing microorganisms.

The antimicrobial activity of apoE-derived AMPs was inhibited by heparin, indicating that the generated AMPs contain heparin-binding motifs. Heparin-binding and antimicrobial motifs share the same features: amphipaticity, cationicity, and secondary structures. Andersson *et al.* reported that synthetic peptides derived from heparin-binding sequences of plasma proteins including e.g. laminin, fibronectin, von Willebrand factor, and vitronectin exerted antimicrobial activity (11). Thus, apoE belongs to a group of heparin-binding plasma proteins that are precursors for AMPs.

Antimicrobial assays using synthetic peptides encompassing the heparin-binding sites of apoE were carried out to locate the antimicrobial motif. SHL14 derived from the heparin-binding sequence of the N-terminal LDL-receptor-binding domain exhibited bactericidal effects against *S. aureus* and *E. coli*. By contrast, WGE23, a peptide derived from the heparin-binding motif of the apoE lipid-binding domain, had no bactericidal effect. Thus, not all heparin-binding sites confer antimicrobial activity and all apoE-derived AMPs contain the heparin-binding sites of the N-terminal domain.

SHL14 antibacterial activity is in agreement with Azuma *et al.*, who reported bactericidal effects of a 30 amino acid peptide derived from the LDL-receptor-binding site (here referred to as LRV30) (74). SHL14, in contrast to LRV30 (74), was not further cleaved by HNE, indicating that proteolysis of apoE by HNE generates SHL14. The apoE-derived AMP shares features of other α-helical AMPs (18): it contains 6 positively charged lysine and arginine residues, no aromatic amino acids, has an isoelectric point (pI) of 12, and possesses an amphipathic

structure as demonstrated in the helical wheel projection and the space-filling model of the peptide.

4.1.2 ApoE enhances phagocytosis

ApoE bound to the surface of *S. aureus* as demonstrated by flow cytometry and serum absorption assays. These results are in line with the findings of Dreisbach *et al*., who characterized the binding of human serum proteins to *S. aureus* by surface shaving (77). However, the functional role of apoE binding to bacteria was unknown.

Here, it is demonstrated that apoE enhances phagocytosis of *S. aureus* by macrophages. The opsonic activity of apoE was dose-dependent. Previously, Grainger *et al.* concluded that apoE modulates the clearance of apoptotic bodies (72). Apoptotic bodies and microorganisms are often recognized by the same opsonins including complement factors (29) and members of the pentraxin family, such as CRP (133-136). Thus, apoE acts like a bridging molecule (23) that binds to pathogens, as well as apoptotic bodies, and initiates phagocytosis. However, the apoE-ligand on the staphylococcal surface is unknown. As apoE binds and neutralizes the PAMP LPS on Gram-negative bacteria (137), it is likely that apoE also recognizes PAMPS on *S. aureus*. ApoE interacts with LDL- and LRP-receptors, which are both implicated in phagocytosis of apoptotic cells (138), and may therefore participate in the apoE-mediated opsonization and clearance of *S. aureus*.

The AMP SHL14, in contrast to full-length apoE, did not enhance phagocytosis; hence antimicrobial activity is independent from opsonic activity. However, several serum proteins such as thrombospondin, vitronectin, fibronectin, and β2-glycoprotein I, interact with macrophage receptors, promote phagocytosis (24, 25), and also harbor antimicrobial sequences (10, 11, 127).

Taken together, apoE (i) exerts antimicrobial activity after its proteolytic processing and (ii) facilitates phagocytosis (FIG. 39). Thus, the increased susceptibility to bacterial infections in mice lacking apoE (67-69) may also be due to reduced antimicrobial and opsonic activity.

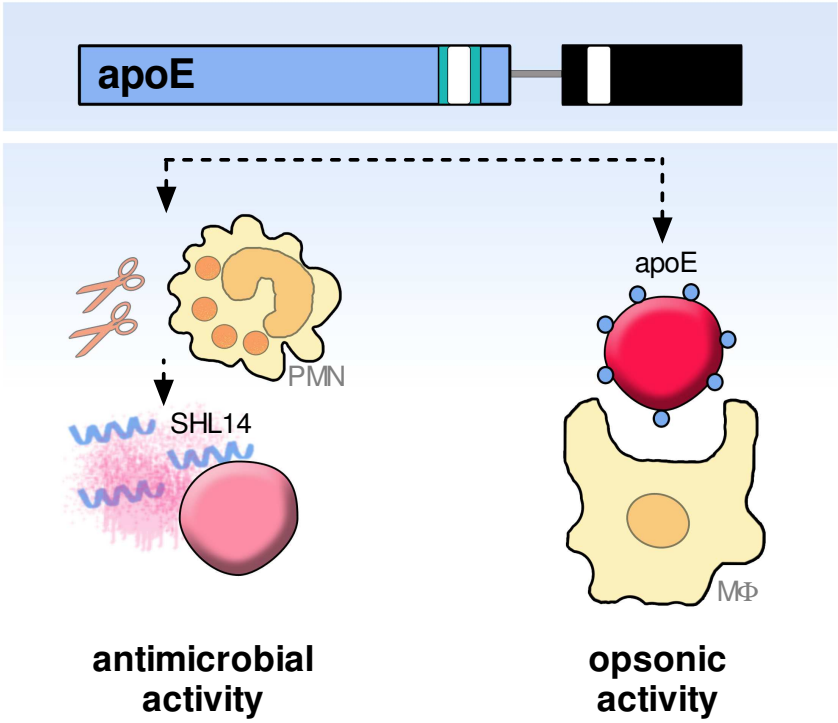

FIG. 39: ApoE has antimicrobial and opsonic activities.
HNE-mediated proteolysis of apoE yields AMPs (left side). ApoE binds and facilitates phagocytosis of *S. aureus* (right side). PMN = polymorphic neutrophil, MΦ = macrophage.

4.2 The role of plasmin during and after infections

Plasmin has been described as a complement activator, because it cleaves C3 generating functionally active C3a (52, 132, 139, 140). However, as reported in this thesis as well as by Barthel *et al.,* and Seya *et al.*, plasmin also has complement inhibitory functions (51, 54). C3 degradation assays revealed that plasmin degrades C3, C3b, and in contrast to factor I and factor H, also iC3b as well as C3c. These observations are in agreement with Nagasawa *et al.*, who reported rapid degradation of iC3b (C3b´) by plasmin into C3c and C3d (53) and Barthel *et al.*, who showed similar C3b cleavage fragments (51).

Plasmin- and factor I-mediated cleavage of C3 and C3b acted in concert and caused rapid degradation of both complement components. Therefore, plasmin, alone or together with factor I, interferes with C3b effector functions by inhibiting the alternative pathway amplification loop, C3b-mediated opsonization, and the propagation of the complement cascade. Consequently, when plasmin is generated it can act together with factor I to protect self cells and tissues from damage by complement activity (51, 141).

Moreover, plasmin degraded AMPs, such as C3a and the apoE-derived SHL14. C3a degradation concurs with Amara *et al.*, who observed while plasmin

generates C3a, the C3a amount decreased with increasing plasmin concentrations (52). Thus, dependent on the amount of plasmin, this protease generates C3a and acts as a complement activator or inactivates the complement effector molecules C3a and C3b to inhibit complement activity. The plasmin level is tightly regulated by inhibition of plasminogen activators and plasmin (57, 142). The fibrinolytic protease primarily acts after coagulation and inflammation and may therefore inactivate no longer needed AMPs after the immune reaction (FIG. 40).

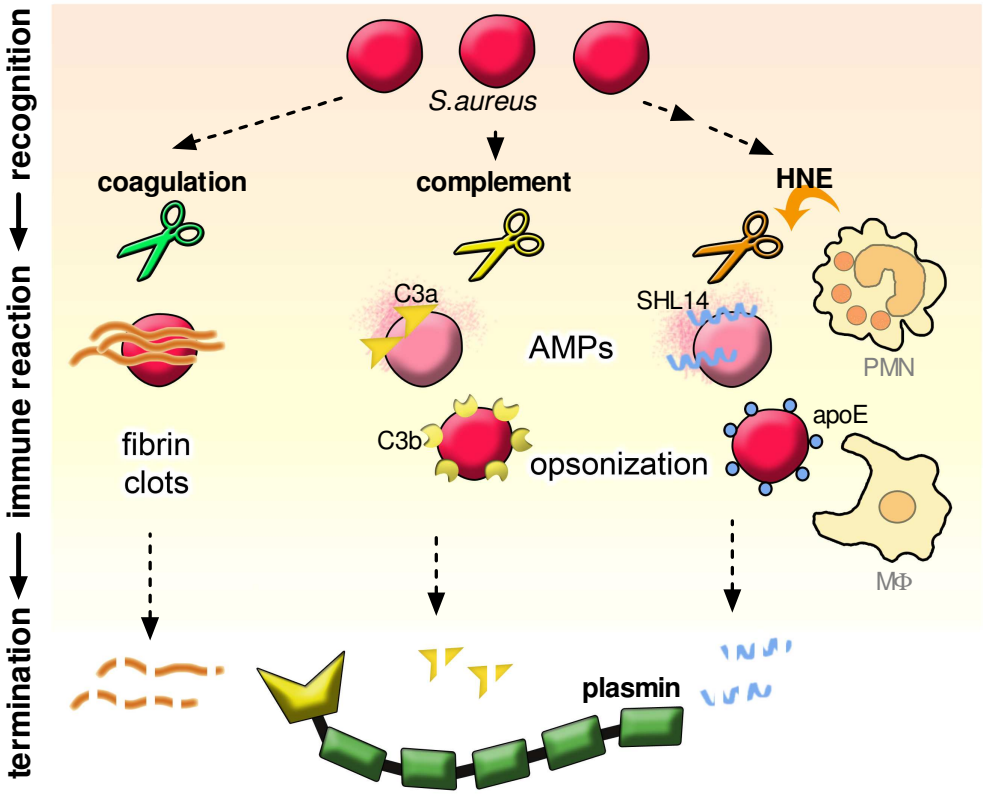

FIG. 40: Plasmin and apoE functions.
Microbial intruders activate the coagulation system leading to generation of fibrin clots that trap microorganisms. Additionally, the complement system is activated resulting in the generation of antimicrobial peptides, such as C3a and C4a, opsonins, and chemo-attractant molecules, which recruit neutrophils and macrophages to infectious sites. Neutrophils secrete proteases that generate AMPs like SHL14. Phagocytosis of microorganisms is facilitated by apoE and C3b/iC3b. After the immune reaction, plasmin degrades fibrin clots and AMPs.

4.3 Staphylococcal innate immune evasion by proteases

As part of the evolutionary “arms race”, microbes, such as *S. aureus,* have developed elaborated evasion strategies to escape the host immune defense. Here, it is reported that *S. aureus* recruits human plasminogen by Sbi and Efb and subsequently activates the serine protease plasmin. Sbi and Efb were shown to

simultaneously bind plasmin together with C3 or C3b and to accelerate the degradation of both complement proteins representing a new type of complement evasion strategy. In addition, *S. aureus* exploited the proteolytic activity of plasmin and the staphylococcal metalloprotease aureolysin to evade cell damaging by AMPs and opsonin-dependent phagocytosis.

4.3.1 Two novel staphylococcal plasminogen-binding proteins

Human plasminogen, the zymogen of plasmin, was recruited by the staphylococcal complement inhibitory proteins Sbi and Efb. Hijacking plasminogen is also reported for several other microbial proteins, summarized in TAB. 1, and reflects a major evasion strategy used by a vast number of pathogens.

The binding of plasminogen to Sbi and Efb was dose dependent and influenced by the lysine analogue ε-ACA. This represents the same method of plasminogen binding observed in the listed microbial proteins and indicates a similar binding strategy to ensure activation of plasminogen to plasmin.

TAB. 1: Plasminogen-binding proteins from human pathogenic bacteria.
The bacterial plasminogen activators are not included.

PLG-binding protein	Abbreviation	Species	Refs
α-enolase		*Bacillus anthracis*	(143)
Elongation factor-tu	EF-tu		(144)
Chaperonin-60 kDa	GroEL		
Borrelia plasminogen-binding protein	BPBP	*Borrelia burgdorferi*	(145)
Outer surface protein A	OspA		(146)
Outer surface protein C	OspC		(147)
Complement regulator-acquiring surface protein 1;3-5	CRASP-1		(148)
	CRASP-3/ErpP		(130)
	CRASP-4/ErpA		
	CRASP-5/ErpC		
α-enolase			(149)
Flagella		*Escherichia coli*	(150)
G-Fimbriae			(151)
Aspertase		*Haemophilus influenzae*	(152)
Protein E	PE		(153)
Plasminogen-binding protein A	pgbA	*Helicobacter pylori*	(154)
Plasminogen-binding protein B	pgbB		
Leptospira endostatin-like protein A	LenA	*Leptospira interrogans*	(155)
8 outer membrane proteins	e.g.: LipL32, Lp29,		(156)

	MPL36, rLIC12730…		
α-endolase Hsp70 chaperone Peroxiredoxin	DnaK	*Neisseria meningitidis*	(157)
Elongation factor Tuf	Tuf	*Pseudomonas aeruginosa*	(158)
Fimbriae		*Salmonella enterica*	(151)
staphylococcal binder of IgG **extracellular fibrinogen-binding protein**	**Sbi** **Efb**		**this study**
α-enolase Ribonucleotide reductase		*Staphylococcus aureus*	(117)
Inosine 5′-monophosphate Dehydrogenase	IMPDH		
Glycerinaldehyd-3-phosphat-dehydrogenase	GAPDH		(159)
α-enolase	Eno	*Streptococcus pneumoniae*	(160)
Cholin-binding protein E	CBPE		(161)
Plasminogen- & fibronectin-binding protein B	PfbB		(162)
Glycerinaldehyd-3-phosphat-dehydrogenase	GAPDH/ SDH		
α-enolase	SEN	*Streptococcus pyogenes*	(163)
Plasminogen-binding group A streptococcal M protein	PAM		(164)

In contrast to previously identified plasminogen ligands, Sbi and Efb bind C3 in addition to plasminogen. Plasminogen binding was mediated by the domains Sbi 3-4 in Sbi and Efb-C in Efb, the same domains that interact with C3. Although the sequence identity between Sbi 3-4 and Efb-C is rather low (only 19%), both fragments contain a three-helix bundle motif, in which the amino acids of the α2-helixes contribute to C3d binding (103, 106).

Sbi 3-4 and Efb-C simultaneously recruited plasminogen and C3 and formed a trimeric complex. In these complexes, plasminogen remained accessible for the staphylococcal activator SAK and the human activator uPa to be converted to plasmin. More than 67% of *S. aureus* strains express the *sak* gene and produce the nonproteolytic SAK (121). In addition, the pathogen can enhance uPa production in mammalian cells (165) or activate conversion of pro-uPa to uPa by the metalloprotease aureolysin (112) to enhance plasmin generation. These different plasminogen activation strategies underline the importance of plasmin

activation, and may explain why staphylococcal strains incapable of expressing SAK showed no loss of virulence (166).

Sbi and Efb are surface exposed and detected in extracellular fractions (95-98). Thus, complexes of plasmin(ogen), C3, and Sbi or Efb are presumably formed on the cell surface and in fluid phase. Conclusively, *S. aureus* binds plasminogen by a number of proteins and activates plasmin by several strategies to exploit the proteolytic activity of the serine protease (FIG. 42).

4.3.2 Proteolytic inactivation of complement C3

Plasmin complexed with Sbi or Efb and either C3 or C3b degraded both complement components, respectively. Interestingly, plasmin-mediated C3 or C3b degradation was accelerated in the presence of Sbi or Efb. These functions were mediated by the C3-binding domains Sbi 3-4 and Efb-C, and concur with studies showing that Efb-C binding to C3 and C3b leads to conformational changes in C3 as well as in C3b (100, 101). Sbi acts similar to Efb and induces structural changes in C3, as predicted in the doctoral thesis of M. Reuter (167), and C3b.

The presented results provided further information about Efb-C functions. This protein affects the structural conformations of C3 and C3b, but not iC3b, because iC3b degradation was not influenced. In addition, the degradation-enhancing effect of Efb-C was also observed for degradation of C3 and C3b by aureolysin. Thus, Efb-C induces an open conformation of C3 and C3b, which increases the accessibility of cleavage sites for proteolysis within the complement molecules. In contrast to C3 and C3b, iC3b already displays an open conformation and therefore its cleavage is not modified. The initial conformational change in C3 presumably leads to a C3(H_2O)-like conformation, as factor I and factor H cleave C3(H_2O), but not C3 (31, 168). Additionally, plasmin degrades iC3b more efficiently compared to C3b (53), thus the second conformational change may lead to an iC3b-like conformation of C3b (FIG. 41).

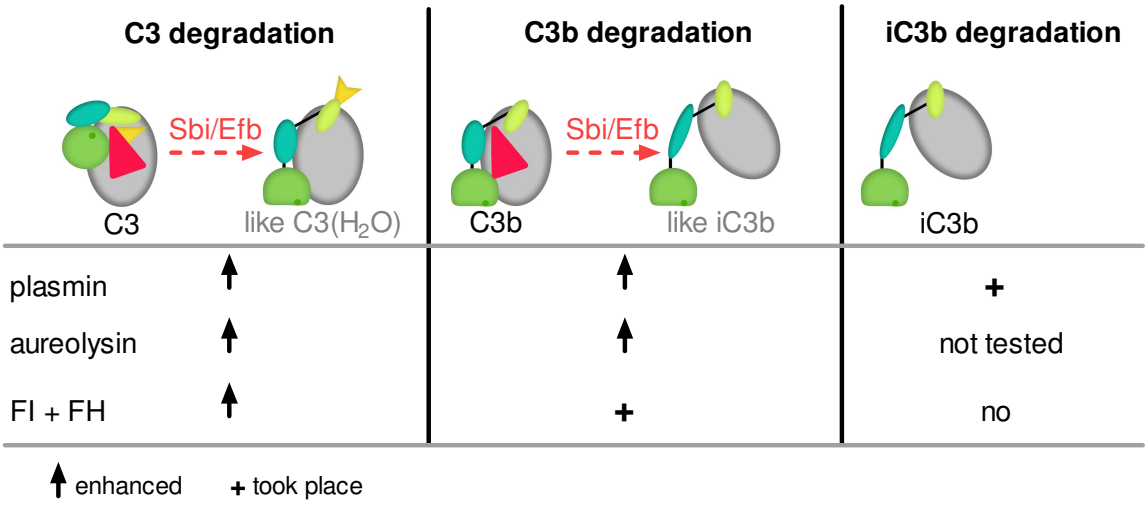

FIG. 41: Proposed model for Sbi and Efb functions.
Sbi and Efb induce conformational changes in C3 and C3b, but not iC3b (first row). These changes accelerate the susceptibility of C3 and C3b to proteolytic degradation by plasmin, aureolysin, and factor I (FI) (second row).

The degradation-enhancing effect of Efb-C was more pronounced, compared to Sbi 3-4. This difference is presumably due to the higher affinity of Efb-C for C3 or C3b. Clark *et al.* also reported that Sbi 4 (Sbi IV), in addition to the overlapping binding site for Efb-C, has another unique binding site for C3d that influences the interaction (103).

Taken together, Sbi 3-4 and Efb-C have both, redundant and distinct functions. The two staphylococcal proteins bind to C3d containing C3 fragments and accelerate the degradation of C3 and C3b by plasmin and aureolysin. This mechanism represents a novel type of complement evasion strategy and is, to our knowledge, unique for *S. aureus*. In addition, Sbi and Efb were recently shown to form tripartite complexes with factor H and inactivate C3 and C3b by factor I ((167) (90, 167). Both staphylococcal proteins also competitively inhibited the binding of C3d to CR2 (104, 105). However, Sbi induces consumption of C3, whereas Efb blocks all C3b-containing convertases (95, 100-102).

S. aureus used the proteolytic activity of plasmin to inactivate C3 and its active cleavage products C3a and C3b. C3a degradation counteracted C3a antimicrobial activities and may also prevent C3a-mediated recruitment of neutrophils to infectious sites. Degradation of C3b, which is accelerated by Sbi or Efb, prevented opsonization and consequently phagocytosis as reported by Rooijakkers *et al.* (116). Thus, extensive degradation of C3 by plasmin leads to C3 consumption without generating functionally active C3a and C3b. A similar consumption effect

was described for the secreted metalloprotease aureolysin. The metalloprotease degrades C3 similarly to the C3 convertase yielding C3a and C3b, which are both inactivated in the presence of human plasma (113). In contrast to the aureolysin-mediated cleavage pattern of C3 reported by Laarman *et al.* (113), we observed additional C3 cleavage fragments, which may be due to higher protease concentrations.

In summary, *S. aureus* exploits plasmin to proteolytically inactivate complement components, such as C3, C3a, C3b, as well as C5 (51) and IgGs (116), fibrinogen and other components of the ECM (43) (FIG. 42). These powerful inhibitory effects of acquired plasmin against several immune defense mechanisms apparently explain why a multitude of pathogenic microbes attach human plasminogen to their surface and activate plasminogen to plasmin.

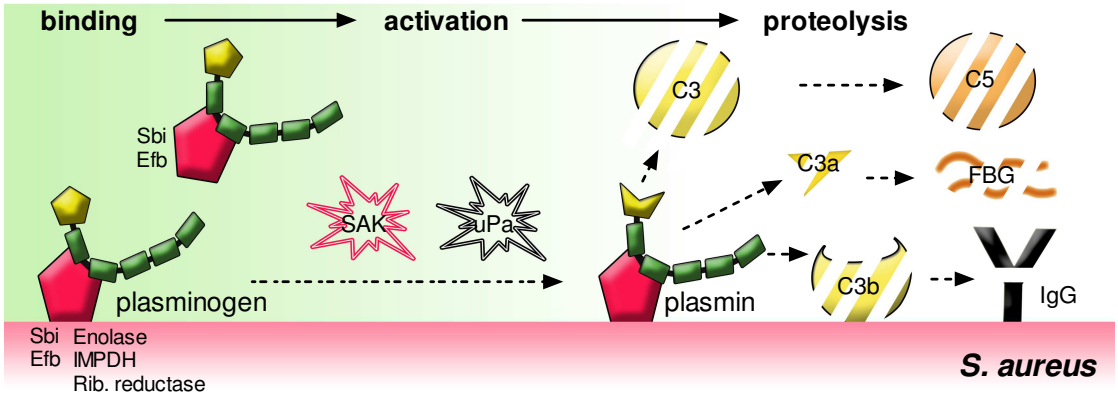

FIG. 42: *S. aureus* binds and activates plasminogen to plasmin for immune evasion. Plasminogen is recruited by surface bound and secreted proteins of *S. aureus*. The zymogen is activated by SAK or the host activator uPa to plasmin. Plasmin then degrades the complement components C3, C3a, C3b, and C5, as well as FBG, and IgG.

4.3.3 Proteolytic inactivation of apoE-mediated immune reactions

In addition to the complement system, *S. aureus* has to overcome further innate immune responses such as apoE-mediated immune reactions (4.1). Here, we demonstrated that *S. aureus* also utilizes host-derived plasmin and aureolysin to inhibit the immunoregulatory activity of apoE.

Proteolytic processing of apoE by HNE yielded AMPs containing SHL14 (4.1.1). However, plasmin and the metalloprotease aureolysin completely degraded the apoE-derived AMP. Degradation of SHL14 by both proteases impaired surface binding and antimicrobial activity. Proteolytic inactivation of α-helical AMPs is in agreement with Sieprawska-Lupa *et al.,* who showed that a similar amount of

aureolysin degrades and inactivates α-helical AMP LL-37. In addition, aureolysin-overexpressing strains were less affected by the anti-staphylococcal activity of LL-37 indicating that the expression level of aureolysin directly affects resistance to α-helical AMPs (111).

Besides SHL14, plasmin and aureolysin also degraded full-length apoE. Two different cleavage patterns of apoE were observed. The serine protease plasmin-mediated degradation of apoE yielded a variety of fragments, whereas the metalloprotease aureolysin cleaved apoE into two distinct fragments (limited proteolysis). Similarly, plasmin degrades C3 into several fragments, while aureolysin cleaves C3 at distinct sites (113). Degraded apoE lost its ability to bind to *S. aureus* leading to reduced apoE-mediated phagocytosis by macrophages. However, an additional, direct effect of plasmin and aureolysin on phagocytosis was observed, as both proteases affected the level of basal phagocytosis.

In summary, aureolysin and plasmin use different cleavage mechanisms, but eventually inactivate SHL14 and apoE. Hence, both proteases block the antimicrobial activity of α-helical AMPs and apoE-mediated opsonization for phagocytosis.

S. aureus also pursues other evasion strategies against cationic AMPs and opsonins. For example, the bacterium modifies its membrane structure or surface net charge to impair recognition by AMPs and thus becomes more resistant (19). Furthermore, SAK itself acts as a trapping molecule for the α-defensins HNP-1 and -2 and neutralizes their bactericidal activity (123). *S. aureus* resists phagocytosis using various secreted molecules. In order to avoid the classical opsonin IgG, the bacterium expresses the IgG-binding proteins Sbi, SpA, and SSL10, which block the Fc-region of IgGs for recognition by Fc-receptors on phagocytes (82-85). Also the complement-derived opsonin C3b is targeted by various soluble staphylococcal inhibitors, such as SCIN (89).

4.3.4 Staphylococcal innate immune evasion strategies

S. aureus uses a variety of secreted molecules to counteract the human immune defense. Remarkably, some of these proteins have overlapping activities. In this thesis, it is shown that plasminogen is bound by several staphylococcal proteins including Sbi, as well as Efb, and subsequently activated by bacterial or human activators to plasmin. Plasmin and the staphylococcal metalloprotease aureolysin have overlapping substrates. Both proteolytically inactivate C3, apoE, and the

apoE-derived AMP SHL14. In addition, Sbi and Efb also share redundant features, such as modification of the C3 and C3b structure. This high level of redundancy may partially explain why *S. aureus* is very successful in immune evasion and difficult to treat. For example, inhibition of aureolysin would have no total effect on the proteolysis of AMPs and opsonins, as plasmin has similar activity.

Another striking fact regarding staphylococcal immune evasion is the accumulation of C3 inhibitors. Targeting the central complement component C3 is especially effective as it inhibits (i) the auto-amplification loop of the alternative pathway, (ii) the generation of AMPs (C3a and C4a) and chemoattractants (C3a and C5a), (iii) the opsonization by C3b, and (iv) the activation of B-cells via CR2. *S. aureus* targets C3 by several secreted proteins, such as SCIN (89), Efb (98), Sbi (95), and aureolysin (113). Additionally, the bacterium hijacks human complement regulators like factor H (90) and the protease plasmin (117). Intriguingly, *S. aureus* combines different strategies and multiplies thereby the inhibitory activities. For example, it is shown in this thesis that proteolysis of C3 is accelerated by Sbi and Efb.

4.4 Concluding remarks

Both human plasma proteins, apoE and plasmin(ogen), interact with the staphylococcal surface. However, the results of this thesis demonstrate that both proteins play distinguished roles in the host-pathogen interactions. As apoE-derived peptides exhibited antistaphylococcal activity and the intact protein facilitated phagocytosis, apoE is part of the innate immune response against *S. aureus*.

By contrast, the activated serine protease plasmin has ambiguous functions that restrict the innate immune defense; it (i) acts as inhibitor of the complement cascade by consuming C3, (ii) terminates the activity of AMPs, (iii) controls coagulation by cleavage of fibrin clots, and (iv) facilitates migration by degradation of components of the ECM (39, 43, 44). Therefore, *S. aureus* recruits plasminogen, activates it to plasmin and exploits the proteolytic activity of plasmin to counteract the immune defense and establish in the host.

The results presented in this thesis enhance our knowledge of the staphylococcal pathogenicity and may help to resolve the mode of action of this clinically relevant bacterium.

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6 APPENDIX

6.1 EIGENSTÄNDIGKEITSERKLÄRUNG

Hiermit erkläre ich,

• dass mir die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller Universität bekannt ist.

• dass die Promotionsarbeit von mir selbst angefertigt wurde, keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen wurden, und alle Hilfsmittel und Quellen angegeben wurden.

• dass alle Personen benannt wurden, die an der Auswahl, Erstellung und Auswertung der Manuskripte beteiligt waren.

• dass die Hilfe eines Promotionsberater nicht in Anspruch genommen wurde und Dritte weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten habe, die im Zusammenhang mit dem Inhalt der Dissertation stehen.

• dass die vorgelegte Dissertation nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht wurde.

• dass weder die gleiche noch eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung bei einer anderen Hochschule als Dissertation eingereicht wurde.

Jena, den 02.07.2012

Tina Katrin Koch

6.2 LEBENSLAUF

Name: **Tina Katrin Koch**, geb. Enghardt

geboren am: 10. November 1984 in Rochlitz

Hochschulausbildung

01/2009 – 10/2012 Promotion am Leibniz-Institut für Naturstoff-Forschung und Infektionsbiologie Jena in der Abteilung Infektionsbiologie unter der Leitung von PD Dr. Christine Skerka, gefördert durch die International Leibniz Research School (ILRS Jena)

Thema der Promotion: „Novel evasion strategies of *Staphylococcus aureus* against the human innate immune response“

10/2003 - 12/2008 Studium der Biologie an der Friedrich-Schiller Universität Jena mit Hauptfach Biochemie und den Nebenfächern Immunologie und Medizinische Mikrobiologie

03/2008 - 12/2008 Diplomarbeit am Leibniz-Institut für Naturstoff-Forschung und Infektionsbiologie, Jena in der Abteilung Infektionsbiologie unter der Betreuung von PD Dr. Christine Skerka

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01/2007 - 06/2007 Auslandssemester an der Umeå University (Schweden)
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Schulausbildung

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Karl-von-Frisch Abiturientenpreis

1995 – 2001 Besuch des Gymnasiums in Penig

6.3 ÜBERSICHT der Vorträge, Poster, Veröffentlichungen und Preise

Vorträge:

5th Annual ILRS Symposium, Jena 2012

***Staphylococcus aureus* blocks innate immune attack by apolipoprotein E generated antibacterial peptides.**

Tina Katrin Enghardt, C. Skerka

XI International Symposium on tick-borne diseases, Weimar 2011

Human complement-related protein 5 and plasminogen are recruited by *B. burgdoferi.*

Tina Katrin Enghardt, P. Kraizcy, P. F. Zipfel, C. Skerka

4th Annual ILRS Symposium, Dornburg 2011

Human Complement Factor H Related protein 5 (CFHR5) and plasmin inactivate Complement when bound by *Borrelia burgdorferi* surface protein CRASP5.

Tina Katrin Enghardt, C. Skerka

ILRS Group Seminar, Jena 2010

Complement evasion of human pathogenic microorganisms.

Tina Katrin Enghardt, C. Skerka

Poster:

63rd. Annual Meeting of the German Society of hygiene and microbiology (DGHM), Essen 2011

The Staphylococcal protein Efb binds plasminogen & enhances plasmin-mediated C3 degradation.

Tina Katrin Enghardt, D. Barthel, M. Reuter, S. Hälbich, P. F. Zipfel, C. Skerka

XXII International Complement Workshop, Basel, Switzerland, 2008

Disease associated protein complement factor H related protein 1 (CFHR-1) is a regulator of the human alternative complement pathway.

S. Heinen, U. Wiehl, N. Lauer, H.-M. Dahse, A. Hartmann, S. Schirmer, Tina Enghardt, R. Wallich, S. Halbich, M. Mihlan, U. Schlotzer-Schrehardt, P. F. Zipfel, C. Skerka

Veröffentlichungen:

***Staphylococcus aureus* Proteins Sbi and Efb Recruit Human Plasmin to Degrade Complement C3 and C3b.**

Tina Katrin Koch, M. Reuter, D. Barthel, S. Böhm, J. van den Elsen, P. Kraiczy, P. F Zipfel, C. Skerka

PLOS ONE, 2012

The role of apoE in the host-pathogen interactions.

Tina Katrin Koch, I. Kopka, H. Nievendick, C. Skerka

(in Bearbeitung)

Factor H related protein 1 (CFHR-1) inhibits complement C5 convertase activity and terminal complex formation.

S. Heinen, A. Hartmann, N. Lauer, U. Wiehl, H.-M. Dahse, S. Schirmer, K. Gropp, Tina Enghardt, R. Wallich, S. Halbich, M. Mihlan, U. Schlotzer-Schrehardt, P. F. Zipfel, C. Skerka

Blood, 2009

Stipendien & Preise:

Preis für den besten Vortrag der ILRS, Jena 2012

***Staphylococcus aureus* blocks innate immune attack by apolipoprotein E generated antibacterial peptides.**

Reisestipendium der DGHM, Essen 2011

The Staphylococcal protein Efb binds plasminogen & enhances plasmin-mediated C3 degradation.

Preis für den besten Vortrag der ILRS, Dornburg 2011

Human Complement Factor H Related protein 5 (CFHR5) and plasmin inactivate Complement when bound by *Borrelia burgdorferi* surface protein CRASP5.

Medac-Forschungspreis 2009 für die Veröffentlichung:

Factor H related protein 1 (CFHR-1) inhibits complement C5 convertase activity and terminal complex formation.

6.4 DANKSAGUNG

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