Immune evasion by Gram-positive pathogens

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Immune evasion by Gram-positive pathogens

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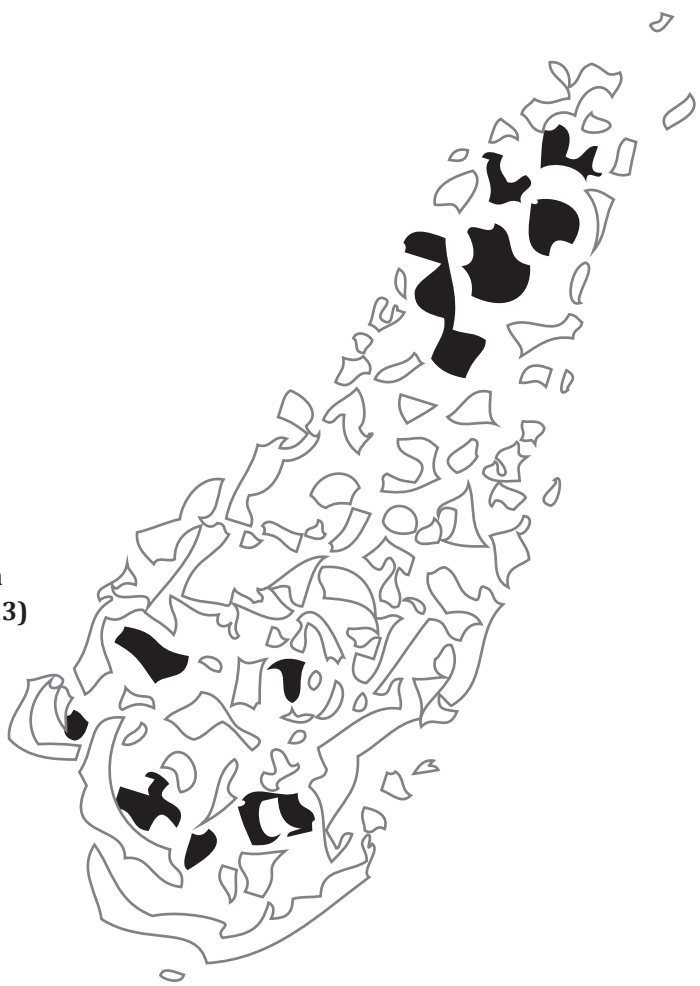

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Innate immunity

Staphylococcus aureus

Staphylococci are microbes well adapted to their host. Of all infections caused by staphylococcal species in human, *S. aureus* is the most common organism isolated and it is the worldwide leading cause of skin and soft tissue infections in general [1]. *S. aureus* is a human commensal, but nasal carriage is associated with an increased risk of infection [2]. Once invaded, *S. aureus* has the capacity to cause a plethora of infections, ranging from cellulitis, erysipelas, abscesses and furuncles to bacteremia and sepsis, endocarditis, osteomyelitis, necrotizing fasciitis and pneumonia [1]. In the 1960s, Methicillin-resistant *S. aureus* (MRSA) was identified as a nosocomial pathogen, affecting hospitalized patients with defined risk factors for acquisition [3]. In the 1990s, infection of previously healthy community-dwelling individuals with MRSA was reported [4]. Since then, community-acquired (CA-) MRSA strains have rapidly emerged worldwide [5]. As a result, interest in the pathophysiology of *S. aureus* has revived. A large arsenal of *S. aureus* virulence factors has been studied, targeting different host immune factors. Notably, *S. aureus* has turned out to be an effective evader of human innate immunity with virulence factors directed towards all its components [6]. A great deal of these factors is directed against neutrophils or against neutrophil functioning.

Neutrophils

Neutrophils comprise 60% of the white blood cell population and are the most important phagocytic cells, defending the host against acute bacterial infection. Patients with congenital neutrophil deficiencies suffer from severe infections that are often fatal [7] and diseases or disease states as agranulocytosis, abnormality of immunoglobulins (Ig) or complement are associated with major bacterial infections. Occasionally, abnormality in neutrophil function is the underlying cause. In such cases, disorders of neutrophil function (such as Chronic Granulomatosis disease, CGD and others) correlate strongly with recurrent cutaneous, periodontal, respiratory, or soft tissue bacterial infections. In many of these cases, *S. aureus* is the causative organism [8,9]. From these studies can be concluded that in healthy individuals neutrophil mediated killing is the key defence system against *S. aureus*.

Indeed, as opposed to other phagocytes as monocytes and macrophages, neutrophils are fully equipped to kill tough Gram-positive bacteria, such as staphylococci. This is achieved by a bombardment with reactive oxygen species in an event described as a “metabolic burst”, in combination with the armamentarium of proteases, antimicrobial peptides and several other enzymes. Neutrophils are therefore the most prominent, if not the only, cells in our immune system that are able to effectively kill staphylococci. However, staphylococci have evolved many ways to resist this killing. First, we will describe the subsequent steps of how neutrophils get to the site of infection and kill microbes in general. Then, the different strategies from *S. aureus* to resist killing or evasion of recognition at all stages will be highlighted.

Extravasation through the endothelium

Once hostile bacteria have successfully invaded, neutrophils have to leave the blood stream

and move to the site of infection. This multistep process, including neutrophil rolling on the endothelial cells, firm adhesion of the neutrophils, diapedesis and chemotactic migration and subsequent killing mechanisms is illustrated in **Figure 1.** Neutrophils need to cross the vascular cell wall to arrive at the site of entry of microorganisms [10].

First, circulating phagocytes, are slowed down near the site of infection. Activated endothelial cells express P-selectin and E-selectin, which interact reversibly with the glycoprotein P-selectin glycoprotein ligand-1 (PSGL-1) on the neutrophil surface. Due to these loosely adhesive contacts between the phagocyte and the endothelial cells, and the shearing force of the flow of blood, the phagocytes "roll" along the vessel wall [11]. The second step is complete arrest of the neutrophils and firmly adherence to the endothelial cells. Firm adhesion depends on the interaction of β2 integrins on the phagocytes, such as LFA-1 and Mac-1, with intercellular adhesion molecule 1 ICAM-1 molecules on the endothelial cells [12]. When neutrophils roll along the endothelium, interaction with chemoattractants, cytokines, bacterial products and selectins activate and cluster the β2 integrins on the surface of the neutrophil resulting in a high affinity interaction and subsequent firm adhesion and crawling of the phagocytes on the endothelial cells [13,14].

Chemical mediators generated during inflammation induce and redistribute a number of complementary "adhesion" molecules present on both endothelial cells and the neutrophil. A complex interaction between the receptors and ligands on both cells results in transmigration through the endothelial junction or even transcellularly through the endothelial cell [15].

The leukocyte recruitment cascade described above is the generally held view of recruitment derived primarily from *in vitro* experiments or easily accessible vessels. Moreover, there is growing evidence that some organs do not use the same paradigm for recruitment. In the liver, for example, neutrophil recruitment is fully dependent on hyaluronan on endothelium and CD44 on neutrophils, independent of selectins [16,17].

Opsonization

Neutrophil phagocytosis is dependent on opsonization of the target microbe via either components of the complement system, Ig or other innate immune components. Complement can rapidly recognize and opsonize bacteria or kill Gram-negative bacteria directly by formation of the membrane attack complex. The human complement system consists of more than thirty proteins and uses three independent pathways to distinguish bacteria from host cells. [18]. The lectin pathway recognizes distinct evolutionary conserved structures such as polysaccharides on microbial surfaces [19]. The classical pathway is initiated by antibody recognition of the microbial target and binding of C1q to the antigen-antibody complex. The alternative pathway is spontaneously activated by hydrolysis of C3 and is generally regarded as amplification loop for C3 cleavage. Host cells are protected by complement inhibitory receptors, however foreign surfaces are targeted as they lack such markers. All three pathways converge at the formation of the C3 convertases, enzyme complexes that catalyse the key reaction in complement activation: cleavage of complement protein C3 into C3a and C3b [6,20]. The majority of C3b is further processed into iC3b by factor H and factor I, but the remaining deposited C3b can form new convertases, thereby amplifying the opsonization process. Subsequently, the high local concentrations of C3b induce a shift in substrate specificity of the convertase to complement protein C5. The

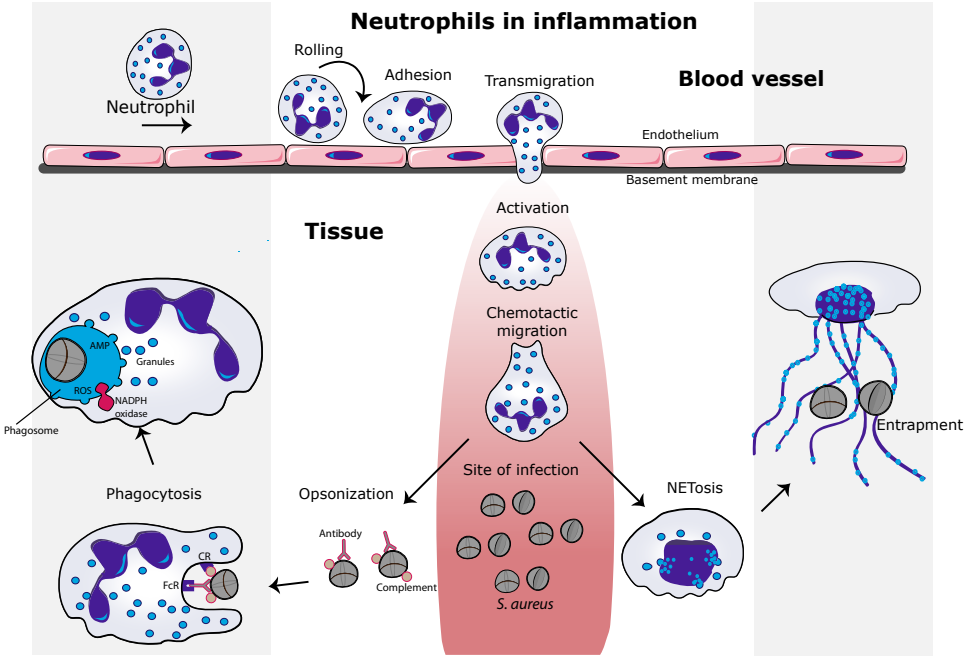

Figure 1. Neutrophil extravasation to site of infection and killing mechanisms. Neutrophil recruitment starts with the detection of signs of inflammation or invading microorganisms by the endothelium. Surveying neutrophils undergo transient interactions with endothelial cells that facilitate neutrophil rolling on the endothelial cell wall and allow neutrophils to come into contact with host- and/or microbe-derived chemotactic signals. Upon stimulation, neutrophils stop rolling and firmly adhere to the endothelial cell wall. This step is followed by neutrophil transmigration through the endothelial wall into the tissue, a process known as extravasation. In the tissue, neutrophils move directed by a chemotactic gradient towards the invading staphylococci, followed by recognition and subsequent phagocytosis. Phagocytosis of *S. aureus* is dependent on the deposition of complement and Ig, which are recognized by complement receptors (CR) and Fcγ receptors (FcR). Inside the neutrophil phagosome, bacteria are killed by antimicrobial proteins (AMP) released upon granule fusion and NADPH oxidase-derived reactive oxygen species (ROS). Alternatively, neutrophils undergo a process called NETosis, whereby neutrophils expel their DNA, decorated with histones and AMP and capable of extracellularly capturing and killing bacteria.

cleavage products of C5 are C5a, one of the most potent chemoattractant, and C5b that initiates the formation of the membrane attack complex and subsequent lysis of Gram-negative bacteria. Together, these complement-mediated events are responsible for the efficient detection and elimination of invading bacteria [21].

Ig help to resolve bacterial infections via immobilization, agglutination and opsonization, which allows for their recognition and ingestion by phagocytes, activate the classical complement pathway and neutralize toxins or other bacterial virulence factors. Different subclasses of Ig are distinct in complement activation or Fcγ-receptor (FcγR) triggering. For instance, IgM is, due to its polymeric nature, particularly effective at complement activation leading to opsonization [22]. The four IgG subclasses differ from each other, depending on their effector functions. This difference is related to their structure, notably with respect to the interaction between the variable, antigen-binding Fab-fragments and the constant Fc fragment. In particular, the length and flexibility of the hinge region are different. This probably relates to the higher activity of IgG3 in triggering effector functions,

when compared to the other subclasses. The capacity of the four human IgG subclasses (in monomeric form) to bind C1q decreases in the order: IgG3>IgG1>IgG2>IgG4, whereby IgG4 completely lacks the ability of complement activation. Effective triggering of the two major FcγR on human neutrophils (FcγR II and FcγR III) is mediated by IgG3 and IgG1 both resulting in phagocytosis [23].

Chemotaxis, priming and activation

After crossing the endothelial barrier, the neutrophil is directed towards the site of infection by a plethora of chemoattractants and primed and activated by a variety of inflammatory stimulants. The primary activating substances are those that are of microbial origin. For Staphylococci they consist of ligands for Toll like receptors (TLR) or chemoattractant receptors of the G protein-coupled receptor (GPCR) family. Perhaps the best-known microbe receptors are the TLRs, responsible for recognizing a number of evolutionary conserved structures. TLR ligands of relevance for staphylococcal infections range from bacterial lipoproteins (TLR1, TLR2, TLR6), to bacterial CpG-rich DNA (TLR9) [24]. TLRs are transmembrane glycoproteins and signal through an intracellular Toll/IL-1 receptor (TIR) domain. Ligand-induced dimerization of TLRs is believed to trigger recruitment of MyD88 to the intracellular TIR domains to initiate signalling via the transcription factor NF-κB [25]. Most TLRs form homodimers upon ligand binding. In contrast, TLR2 forms heterodimers with either TLR1 (TLR1/2) or TLR6 (TLR2/6) to adequately respond to tri- and diacylated lipoproteins, respectively [26]. Gram-positive bacteria contain diacylated lipoproteins, whereas lipoproteins of Gram-negative bacteria have an additional acyl group. Staphylococcal strains deficient in diacyl modification of lipoproteins fail to activate TLR2 *in vivo*, resulting in a reduced inflammatory response [27]. Cells become activated and primed after the activation by TLRs. TLRs assist in phagocytosis, but not involved in chemotaxis.

Neutrophils are attracted to the site of infection through sensing of chemotactic factor gradients [28]. Chemoattractants are secreted by activated host cells or released as activated complement components. All these chemoattractants activate phagocytes by binding to membrane-bound receptors that belong to the GPCR superfamily.

Newly synthesized bacterial proteins contain a formylated methionine and consequently bacteria secrete a quite substantial amount of N-formylated proteins and peptides. fMLP, the prototype N-formyl peptide, induces and potentiates chemotaxis, phagocytosis, and the generation of oxidative burst in neutrophils and monocytes. Formylated peptides act on the formyl peptide receptor (FPR1) and its homologue FPR2, both belonging to the GPCR family. FPR1 binds fMLP with high affinity, while FRP2 shows low affinity for the ligand. Neutrophils express FPR1 and FPR2 but not FPR3, while monocytes express all three GPCRs [29].

Formyl peptides are not the only chemoattractants produced by staphylococci. Phenol -soluble modulins (PSMs) were first described as lytic molecules for neutrophils [30]. Next to lysing neutrophils, PSMs also activate and attract leukocytes via FPR2 [31].

Probably the most potent chemoattractant, next to fMLP, is the small complement fragment C5a. C5a and its less potent counterpart C3a, also commonly called anaphylatoxins, are generated during the activation of the complement cascade. The formation of the small cleavage products C3a and C5a plays an important role in the attraction of phagocytes to the site of infection and the priming and activation of phagocytes for a proper response

against Gram-positive bacteria [32]. Besides bacterial fragments and complement-derived activation products, chemokines and other lipid mediators make neutrophils arrive at the site of infection [33].

Phagocytosis

S. aureus opsonised by complement and/or antibodies are rapidly phagocytosed, when a neutrophil comes in contact with them. Opsonin-coated microorganisms bind to specific receptors on the surface of the phagocyte and invagination of the cell membrane occurs with the incorporation of the microorganism into an intracellular phagosome [34]. Uptake of the bacteria is aided by factors such as C5a, TLR ligands on the surface of the bacterium and ligands for C-type lectin transmembrane proteins such as DC-SIGN, Dectin-1 and the mannose receptor [35]. However, phagocytosis is primarily stimulated by opsonin receptors expressed on the cellular membrane. Receptors for these opsonins, respectively the leukocyte Fc gamma receptors (FcγR) and the complement receptors (CR), recognize Ig and complement factors. Upon cross linking by ligand binding, these receptors induce the initial uptake or endocytosis of the pathogen, whereupon transport to lysosomes and intracellular killing is executed.

FcγRs are members of the Ig superfamily. They contain a ligand binding chain consisting of two or three Ig-like domains, a transmembrane region, and an intracellular tail. Through binding of the constant domain of IgG they provide specificity to immune effector cells. Cross linking of FcγR by IgG-opsonized particles or immune complexes induces several cell type dependent effector functions, including phagocytosis, respiratory burst, cytotoxicity, degranulation, and secretion of inflammatory mediators. Based on structural and biochemical differences they are divided into three distinct classes, FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16), containing twelve isoforms that differ in cell distribution and affinity for IgG subclasses [36]. FcγRIIa is the predominant receptor in phagocytosis. Most FcγR are activating receptors and possess immunoreceptor tyrosine based activation motifs (ITAMs), however FcγRIIb is an inhibitory FcγR that bears an immunoreceptor tyrosine-based inhibition motif (ITIM) in its intracytoplasmic domain. Exceptionally, FcγRIIIb is inserted in the outer layer of the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor and therefore contains no signalling motif [37].

Complement receptor type 1 (CR1, CD35) binds to the active complement fragment C3b, resulting in phagocytic uptake. However CR1 is also a complement regulator, with decay accelerating activity. Complement receptor type 3 (CR3, Mac-1, CD11b/CD18) and complement receptor type 4 (CR4, p150/95, CD11c/CD18) are heterodimeric glycoproteins from the integrin family with a shared ß-chain (CD18). Both receptors show specificity for the iC3b fragment, in a Mg2+-dependent fashion. Stimulation of neutrophils and monocytes via CR3 results in phagocytosis, production of ROS and degranulation [38]. After phagocytosis and phagosome formation, the neutrophil is equipped with multiple efficient mechanisms aimed at killing the intracellular bacteria.

NET formation

Next to phagocytosis and intracellular killing by neutrophils, a new antimicrobial strategy has been described in which neutrophils expel their nuclear content together with several

cytosolic and granular proteins. This results in the formation of neutrophil extracellular traps (NETs), which trap pathogens and thereby prevent further dissemination [39]. Although some essential steps in the formation of NETs (NETosis) have been described [40], the exact initiation, downstream activation pathway, and effector functions are still under investigation. *In vitro* activation of NETs can be initiated by different stimuli such as PMA, LPS, IL-8 and different bacteria [41,42]. However, recent data from Yipp *et al* [43], using intravital microscopy, show that *in vivo* neutrophil NETosis is tightly regulated through TLR2 and complement mediated opsonization. NETs can trap microbes *ex vivo* and have been found in various disease models *in vivo* [44]. Normally, extracellular concentrations of antimicrobial peptides (AMPs) are too low to be effective, however in NETs, local concentrations of AMPs are sufficient for microbial killing.

Bacterial killing mechanisms

Neutrophils are end-stage cells that have stored most of their toxic machinery in ready-to-use granules; organelles for safe storage and ideal for the dynamic interplay with microbes during inflammation and microbial killing. Within these granules different molecules are stored that are directly or indirectly linked to microbial killing. Several enzymes target the bacterial cell wall (such as lysozyme), proteinacious virulence factors (different proteases) or assist in the metabolic burst (myeloperoxidase (MPO). A group of directly bactericidal and bacteriostatic proteins and peptides attack primarily the bacterial membrane, such as the bactericidal/permeability-increasing protein (BPI) or interfere with the cell wall synthesis machinery (defensins) [45]. Different granules fulfil different purposes in the complex dynamics involved in migration, phagocytosis and killing. Located near the plasma membrane and very rapidly secreted are the membrane or secretory vesicles. They serve as a reservoir for a number of important membrane-bound molecules and receptors employed during neutrophil migration and the early steps in opsonic recognition. Azurophilic granules contain many directly antimicrobial proteins such as: MPO, BPI, lysozyme, defensins, and the serine proteases: neutrophil elastase (NE), proteinase 3 (PR3), and cathepsin G (CG). Specific (or secondary) granules, contain lactoferrin, NGAL, hCAP-18, and lysozyme. Gelatinase (tertiary) granules, contain a number of metalloproteases, such as gelatinase [7]. Chelating agents interfere with microbial metabolism and are strongly bacteriostatic. Lactoferrin binds preferentially to iron and calprotectin chelates zinc. However, calprotectin is not stored in granules. In neutrophils, as opposed to all other cells, calprotectin constitutes more than 60% of total proteins in the cytosol of neutrophils [46,47].

Neutrophil activation leads to production of reactive oxygen species (ROS), a process known as the oxidative burst. ROS represents a complex and dynamic mixture of highly reactive molecules that differ in their stability, reactivity, and permeability to membranes, but all can damage microbes severely [48]. Within the phagosome, in the small space between an ingested bacterium and the membrane of the phagosome, molecular oxygen is reduced and high levels of superoxide (O_2^-) are generated upon assembly of the NADPH-dependent oxidase. Electrons are pumped into the phagosome which is compensated by an influx of protons or other cations. The protons are used to reduce superoxide to hydrogen peroxide (H_2O_2). H_2O_2 can combine with chloride to form hypochlorous acid (HOCl) in a reaction catalyzed by MPO. In other secondary reactions, hydroxyl radicals, chloramines, hydroperoxyl radicals, peroxynitrite, and singlet oxygen, all very effective anti-microbial compounds can

be generated. Augmentation of the NADPH oxidase by MPO facilitates a highly efficient response against invading microorganisms and this property is unique for neutrophils [49].

Taken together, neutrophils are highly efficient cells that can find, recognize, take-up and destroy *S. aureus*. However, *S. aureus* is not just a defenceless victim in these antibacterial processes. *S. aureus* has evolved multiple strategies to diminish the efficacy of the antibacterial actions of the host. Below, the different ways of how *S. aureus* fights this attack are highlighted in order, to illustrate that every single step in neutrophil defence mechanisms for bacterial uptake and killing is targeted by *S. aureus*. The remainder of this review will provide an update on the strategies *S. aureus* employs to evade, disarm and attack human neutrophils.

Evasion from phagocytes

Evading extravasation

The very first step of extravasation, rolling on endothelial cells, is modulated by staphylococcal superantigen-like 5 (SSL5) by its effect on P-selectin glycoprotein ligand-1 (PSGL-1) [50]. By binding PSGL-1, SSL5 blocks the interaction with its natural ligand P-selectin and abrogates neutrophil rolling on endothelial cells. SSL5 binding to PSGL-1 is glycan dependent. Subsequent data on SSL5 revealed its structure in complex with sialyl Lewis X (sLex) [51], the predominant sugar moiety on PSGL-1. SSL5 scavenges chemokines from the chemokines receptors through other glycoproteins. Chemokines all contain a highly homologous GAG-binding site, which allows their presentation by endothelial cells or extracellular matrix. SSL5 targets the GAG-binding site common among all chemokines and thereby inhibits activation of integrins, important for neutrophil attachment [52].

The next step in neutrophil extravasation, the firm adhesion to the endothelium is also targeted by *S. aureus*. ICAM-1, the crucial molecule in this interaction is bound by extracellular adherence protein (Eap) [53], blocking also the final molecular adherence step in primary rolling and transmigration events in the blood stream near the infectious focus. These modulators are shown in **Figure 2A.**

Evading chemotaxis, priming and activation

S. aureus secretes many proteins that affect chemokine signalling as depicted in **Figure 2B.** In addition to PSGL-1 inhibition, SSL5 inhibits chemokine-induced leukocyte activation in a different way. SSL5 inhibits leukocyte responses to CXC-, CC-, and CX3C-chemokines and to the complement fragments C3a and C5a. SSL5 directly binds the N-termini of chemokines receptors. Its binding was also dependent on the presence of glycans. Indeed, SSL5 also binds to the FPR1, FPR2, leukotriene B4 receptor, platelet-activating factor receptor, and the nucleotide receptor P2Y2. However, SSL5 does not inhibit the stimulation of these receptors. Non-protein stimuli primarily interact with the transmembrane regions of their target GPCR. Chemokines bind the N-terminus of their receptors, and subsequently interact with the pocket formed by the transmembrane domains. SSL5 thus targets the glycosylated N-termini of all GPCRs, but only inhibits stimuli of protein nature that require the receptor

N-terminus for activation [52].

SSL10 inhibits cell responses mediated by the chemokine CXCL12 (or stromal cell-derived factor-1 α, SDF-1α). When cells are treated with SSL10, calcium mobilization and chemotaxis in response to CXCL12 is abrogated. CXCL12-induced intracellular signaling was also affected in SSL10-pretreated cells. SSL10 is specific for CXCR4-mediated responses, as it did not inhibit CXCL8- or C5a-induced calcium responses [54].

Also proteases assist in inhibition of chemokine signalling. Neutrophils treated with Staphopain A are unresponsive to activation by all unique CXCR2 chemokines due to cleavage of the N-terminal domain. Moreover, Staphopain A inhibits neutrophil migration towards CXCR2 chemokines. By comparing a methicillin-resistant *S. aureus* (MRSA) strain with an isogenic Staphopain A mutant, it is demonstrated that Staphopain A is the only secreted protease with activity towards CXCR2. Secretion of Staphopain A at the site of infection is likely to inhibit neutrophil activation and recruitment, adding to the elaborate immune evasion repertoire of *S. aureus* [55].

Chemotaxis inhibitory protein of *S. aureus* (CHIPS), a small protein of 14.1 kDa, binds and inhibits FPR1 as well as the C5aR, thereby affecting neutrophil chemotaxis [56]. The CHIPS-binding site on FPR1 involves multiple regions in the extracellular domains of the receptor. CHIPS probably binds near or directly at the fMLP-binding pocket, since fMLP is a small peptide that would otherwise easily bypass the blockade. The N-terminus of CHIPS is important for its activity towards FPR1 [57].

CHIPS exerts its effect on C5aR through binding to amino acids 10 to 18 in the N-terminus of this receptor [58]. Deletion of the first 30 amino acids did not affect its C5aR-inhibitory activity suggesting two different active sites. The 3D structure of the C5aR-blocking domain of CHIPS (CHIPS31-121) was resolved [56]. CHIPS31-121 is composed of an α-helix packed onto a 4-stranded antiparallel β-sheet. This domain is structurally homologous to the C-terminal domain of SSLs [59].

A search for *S. aureus* proteins homologous to CHIPS led to the identification of FLIPr/FLIPr-L. As outlined above, formylpeptides provide a bacteria-specific broad signature that is sensed by formylpeptide receptors. *S. aureus* has evolved at least two antagonists for the FPRs. Both CHIPS and FLIPr-L inhibit FPR1 thereby avoiding recognition. Via this unique pathway, FLIPr/FLIPr-L also inhibit FPR2 and thus evade recognition of PSMs that are secreted by staphylococci [31].

All these molecules together inhibit migration towards the site of infection by the very first chemoattractants present upon infection. Furthermore, cellular activation, important for co-signalling events during phagocytosis is inhibited. FLIPr/FLIPr-L also impair responses to FPR2 agonists; it inhibits neutrophil calcium mobilization, actin polymerization, and chemotaxis to the synthetic peptides and endogenous proteins serum amyloid A, amyloid β1-42 and Prion protein106-126. In *S. aureus* biofilms PSMs form amyloid-like fibres [60]. Although these fibres are different from the monomeric PSM, it is tempting to speculate that FPR2 is involved in amyloid recognition in general, recognizing a molecular signature in amyloid structures.

Toll-like receptors (TLRs) are crucial for our host defense against microbial infections. TLR2 is especially important as it recognizes bacterial lipoproteins of both Gram-positive and Gram-negative origin together with TLR1 and TLR6. Present on a variety of immune cells,

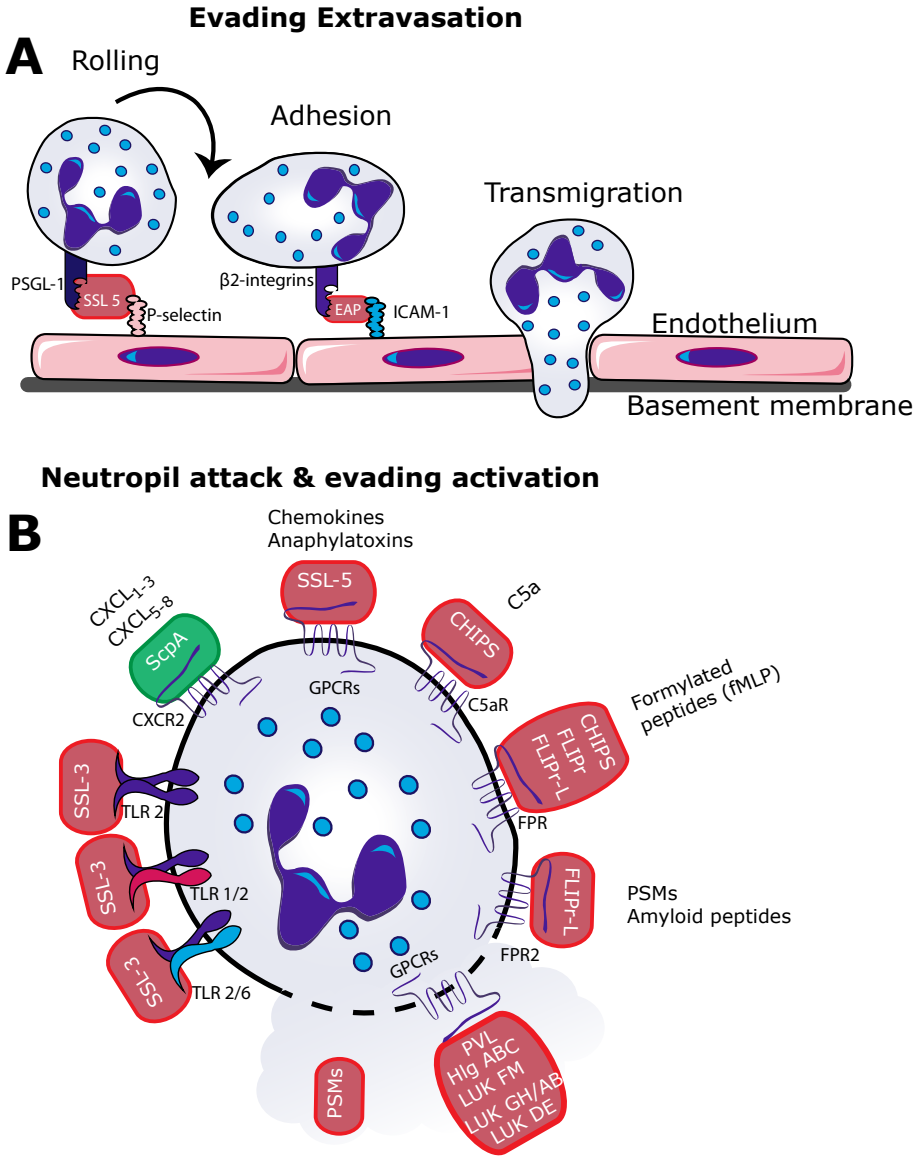

Figure 2. Evasion of neutrophil recruitment, activation, chemotaxis and staphylococcal toxins. (A) Mechanisms by which *S. aureus* subverts neutrophil recruitment and activation. Staphylococcal superantigen-like protein (SSL) 5 antagonizes P-selectin glycoprotein 1 (PSGL-1), extracellular adherence protein (EAP) blocks intracellular adhesion molecule 1 (ICAM-1), and thereby these proteins limit neutrophil recruitment. (B) Chemotaxis or activation inhibitory proteins and mechanism of action of staphylococcal toxins. Red boxes represent an antagonizing protein of *S. aureus*. Green Box represents a protease of *S. aureus*. Abbreviations; Toll like receptor (TLR), G protein-coupled receptors (GPCR), C5a receptor (C5aR), chemokine receptors (CXCR), chemotaxis inhibitory protein of *S. aureus* (CHIPS), formylated protein receptor (FPR), FPR inhibitory protein (FLIPr and FLIPr-L), phenol-soluble modulins (PSMs), Staphopain A (ScpA), leukocidin (LUK), gamma-haemolysin (Hlg) alpha-haemolysin (Hla) and Panton-Valentine leukocidin (PVL).

TLR2 is critical for host protection against *S. aureus*. SSL3 specifically binds and inhibits TLR2 activation on human and murine neutrophils and monocytes. Through binding of the extracellular TLR2 domain, SSL3 inhibits IL-8 production by HEK cells expressing TLR1/2 and TLR2/6 dimers, stimulated with their specific ligands. The SSL3-TLR2 interaction is partially glycan dependent [61,62]. This unique function of SSL3 adds to the arsenal of evasive molecules that *S. aureus* can employ to subvert both innate and adaptive immunity.

Evading opsonization, phagocytosis and NET formation

S. aureus has evolved a whole array of highly specific complement-modulating strategies, as illustrated in **Figure 3**. In this way bacteria can either diminish or delay the detrimental effects of an innate immune attack, thereby creating a window of opportunity to divide and create a microenvironment that allows an even better survival.

The secreted metalloprotease aureolysin attacks the central molecule in the complement system, C3. It effectively inhibits phagocytosis and killing of bacteria by neutrophils. Strikingly and opposed to many other proteases, aureolysin is more potent in serum then in purified conditions. Aureolysin cleaves purified C3 specifically in the α-chain, close to the C3 convertase cleavage site (differs 2 amino acids), yielding active C3a' and C3b'. However, in serum we observe that the aureolysin-generated C3b is further degraded by factor H and factor I. Using an aureolysin mutant in *S. aureus* USA300, it is shown that aureolysin is essential and sufficient for C3 cleavage by bacterial supernatant. Hence, aureolysin acts in synergy with host regulators to inactivate the central complement component C3, thereby effectively dampening the host immune response [63].

Staphylococcal complement inhibitor (SCIN) is a 10 kD, excreted protein that blocks all complement pathways: the lectin, classical and alternative pathway. SCIN (but also SCIN-B and SCIN-C) efficiently prevent phagocytosis, killing of staphylococci and C5a production. SCIN specifically acts on surface-bound C3 convertases, which has two major consequences. First SCIN stabilizes C3bBb at the surface of the bacterium. Secondly and surprisingly, together with the stabilization, the binding of SCIN to C3bBb impairs the enzymatic activity of the convertases [64]. The crystal structure of the C3 convertase formed by C3b, Bb and SCIN was dimeric in nature. SCIN blocks the formation of a productive enzyme-substrate complex [20,65]. Formation of dimeric convertases by SCIN is important for *S. aureus* immune evasion, because it modulates complement recognition by phagocytic receptors. Dimeric, but not monomeric, SCIN convertases showed an impaired binding to complement receptor 1 and the complement receptor of the Ig superfamily. The dimerization site of SCIN is essential for its strong antiphagocytic properties [66].

The extracellular fibrinogen binding molecule (Efb) is a 15.6 kD excreted molecule that was described earlier to bind fibrinogen. Efb binds the C3d region of C3 [67]. Efb and extracellular complement-binding protein (Ecb) can modulate the alternative pathway convertase by binding directly to the C3b molecule [68]. The crystal structures of both molecules in complex with the C3d domain of C3 have revealed their exact binding sites [69]. Targeted inactivation of the genes encoding Ecb and Efb strongly attenuates *S. aureus* virulence in a murine infection model: mice experienced significantly higher mortality rates upon intravenous infection with wild-type bacteria than with an isogenic ΔEcbΔEfb mutant. In addition, Ecb and Efb are both required for staphylococcal persistence in host tissues and abscess formation in the kidneys. In an experimental pneumonia model, Ecb and Efb

together promote bacterial survival and block neutrophil influx into the lungs. Thus, Ecb and Efb are essential to *S. aureus* virulence *in vivo* and could be attractive targets in future vaccine development efforts [70].

Similar to other highly successful invasive bacterial pathogens, *S. aureus* recruits the complement regulatory protein factor H to its surface to inhibit the alternative pathway of complement via surface protein SdrE. Studies using recombinant SdrE revealed that rSdrE bound factor H whether from serum or as a purified form. Furthermore, rSdrE-bound factor H exhibited cofactor functionality for factor I-mediated cleavage of C3b to iC3b [71].

SSL7 binds immunoglobulin A (IgA) and complement C5, thereby inhibiting IgA-FcαRI binding and serum killing of *Escherichia coli* [72]. Furthermore, SSL7 inhibits C5a generation induced by staphylococcal opsonization, which is slightly enhanced by its IgA-binding capacity. SSL7 is strongly protective against staphylococcal clearance in human whole blood. SSL7 inhibited the C5a-induced phagocytosis of *S. aureus* and oxidative burst in an *in vitro* whole-blood inflammation model [73]. The crystal structure of the C5-SSL7 complex confirms that binding to C5 occurs exclusively through the C-terminal beta-grasp domain of SSL7 leaving

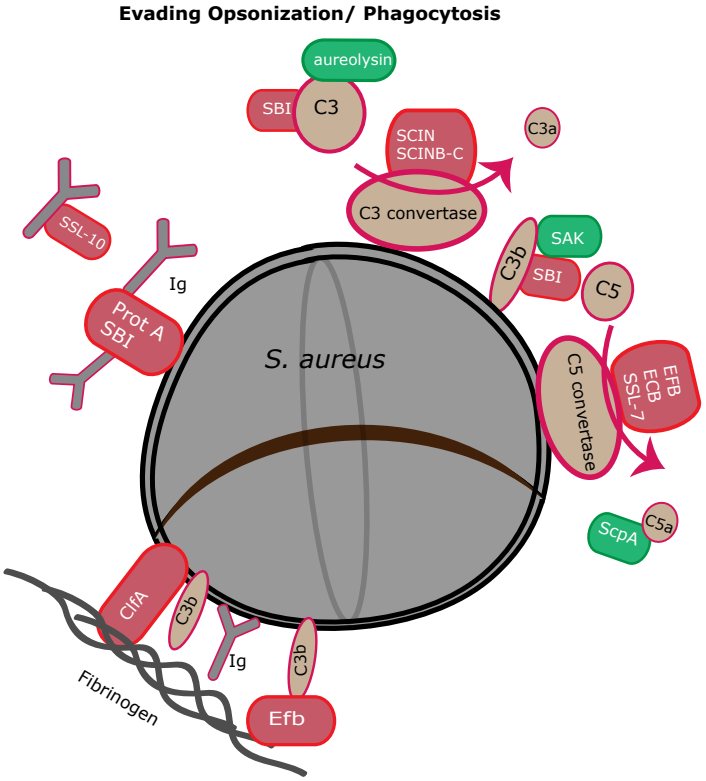

Figure 3. Evasion of opsonization. Schematic representation of the molecular mechanisms of staphylococcal inhibition of opsonization, and subsequent phagocytic uptake by neutrophils. Red boxes indicate an antagonizing protein, green boxes indicate a staphylococcal protease. Abbreviations; Staphylococcal binder of IgG (Sbi), staphylococcal superantigen-like protein (SSL), FPR inhibitory protein (FLIPr and FLIPr-L), immunoglobulin G (IgG), staphylokinase (Sak), staphylococcal complement inhibitor (SCIN), extracellular complement-binding protein (Ecb), extracellular fibrinogen-binding protein (Efb), clumping factor A (ClfA), Fcγ receptors (FcR) and Staphopain A (ScpA).

an distinct domain free to interact with IgA [74].

The first described anti-opsonic molecule of *S. aureus* is staphylococcal protein A (SpA) with its capacity to bind the Fc part of IgG. SpA is a type I membrane protein that is bound to the cell wall of *S. aureus* via its C-terminal cell wall-binding region. SpA is surface-bound, but is released in the surrounding environment during bacterial growth. Through binding the Fc part, protein A blocks Fc-receptor-mediated phagocytosis, but is also a highly efficient complement activation modulator by interfering with binding of C1q. SpA was also described as a B cell "superantigen" promoting B cell activation. Subsequent analysis revealed that surface SpA, similar to B cell receptor cross-linking with anti-human Ig, sensitizes B cells for the recognition of cell wall-associated TLR2-active lipopeptides (LP) [75]. Immunization of mice with SpA, mutated in each of the five Ig-binding domains, raised antibodies that blocked the virulence of staphylococci, promoted opsonophagocytic clearance, and protected mice against challenge with highly virulent MRSA strains [76].

Next to its properties as an CXCR4 antagonist, SSL10 binds IgG1 with consequences for FcR recognition and complement activation. The specific interaction between recombinant SSL10 and human IgG1 was confirmed by Western blot, pull-down analysis and surface plasmon resonance revealing a dissociation equilibrium constant of 220 nM [77].

Two staphylococcal proteins affect both Ig as well as complement. The staphylococcal IgG binding molecule Sbi has two IgG binding domains, with similarity to protein A. Sbi-III and IV can also bind to C3, inhibiting its activation [78]. Furthermore, Sbi binds the human complement regulators factor H and factor H-related proteins and can form a stable tripartite complex with C3 and Factor H [79]. Altogether these actions result in inhibition of the alternative pathway.

Staphylokinase (SAK) targets plasminogen to the staphylococcal surface and activates it into plasmin. Plasmin cleaves human IgG as well as human C3b and iC3b from the bacterial cell wall leading to impaired phagocytosis by human neutrophils. Plasmin removes the entire Fc fragment, thereby inhibiting the activation of the classical pathway of complement and FcR recognition. Plasmin cleaves C3b in both the α- and the β-chain [80]. Clumping factor A (ClfA) binds fibrinogen and blocks phagocytosis by preventing the deposition or recognition of opsonins [81]. Efb may act in a similar manner by binding both C3b and fibrinogen (unpublished data).

All these proteins described above influence opsonisation and therefore directly affect the phagocytic uptake. NET formation is also inhibited by a secreted staphylococcal nuclease. The isogenic nuclease-deficient *S. aureus* mutant was impaired in its ability to degrade NETs *in vitro*. Also, the mutant strain was more susceptible to extracellular killing by activated neutrophils. Moreover, *S. aureus* nuclease production was associated with delayed bacterial clearance in the lung and increased mortality after intranasal infection. Therefore, it is likely that *S. aureus* nuclease promotes resistance against NET-mediated antimicrobial activity of neutrophils and contributes to disease pathogenesis *in vivo* [82].

Evading killing

Most *S. aureus* strains produce a "shield" against ROS that consists of their golden pigment staphyloxantin. This pigment can function as an antioxidant and provides resistance to

killing by H_2O_2 and singlet oxygen. A mutant no longer able to synthesize these carotenoid pigments has an impaired survival inside neutrophils. When the oxidative burst was inhibited by DPI, no difference in killing between the WT and the carotenoid mutant was observed, indicating that the observed effect is directly linked to the ROS activity [83].

Next to the pigment, also several other enzymes can form a defence mechanism against ROS. The main enzyme for removing oxidative stress is intracellular produced catalase. Catalase removes hydrogen peroxide by converting it into oxygen and water, thereby protecting the phagocytosed microbe. *S. aureus* further contains the alkyl hydroperoxide reductase gene (*ahpC)* encoding for an enzyme with catalase activity. Recently, yet another protein is described that also confers resistance to neutrophil killing. This protein, named SOK (surface factor promoting resistance to oxidative killing) is exposed at the extracellular surface. A SOK mutant has an increased sensitivity to singlet oxygen. In an *in vitro* phagocytosis model the WT is more resistant to killing by neutrophils compared to the SOK mutant strain [84].

Importantly, catalase activity provided by either KatA or AhpC is required for normal growth under aerobic conditions [85]. Their additional effects on immune evasion after phagocytosis can be an added value, if not the main function of these enzymes.

Lysozyme will degrade the cell wall peptidoglycan matrix by breaking the ß-1,4 glycosidic bonds between N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc), causing lysis of the bacteria [86]. However, *S. aureus* is insensitive to lysozyme. The O-acetyltransferase A enzyme (OatA) of *S. aureus* causes O-acetylation of the peptidoglycan. Therefore, the muramidase activity of lysozyme is no longer capable of degrading its peptidoglycan [87,88].

S. aureus has at least two independent mechanisms to resist the attack of defensins that disrupt the integrity of bacterial cell wall. First, the cell membrane itself is modified. The underlying mechanism for this resistance is a modification of phosphatidylglycerol with l-lysine. This modification leads to a reduced negative charge of the membrane surface, likely causing repulsion of the cationic peptides. An mprF mutant strain is killed considerably faster by human neutrophils and exhibited attenuated virulence in mice [89,90]. Moreover, *S. aureus* secretes staphylokinase, which activates host plasminogen. Interestingly, a direct binding between α-defensins and staphylokinase results in an almost complete inhibition of the bactericidal effect of α-defensins. Notably, staphylokinase with a blocked plasminogen binding site still retained its ability to neutralize the bactericidal effect of α-defensins [91].

In the resistance against cationic AMPs carotenoid pigments play a role. An increase of the carotenoid in the bacterial membrane makes the membrane more rigid and therefore less susceptible to the AMPs [92].

Aureolysin is capable of cleaving the antimicrobial peptide LL-37, which is one of the few AMPs with potent activity against staphylococci [93].

Finally, *S. aureus* resistance against AMPs is mediated by positively charged modifications of the cell wall through the incorporation of cell wall teichoic acids and lipoteichoic acids [94]. An overview of these protective factors is provided in **Figure 4.**

Neutrophil attack

A hallmark feature of *S. aureus* is its ability to destroy host cells by damaging the cell membranes as shown in **Figure 2B.** By secreting cytolytic toxins, *S. aureus* protects itself against killing by the host immune system both before and after engulfment by neutrophils.

β-barrel pore-forming toxins target the cell membrane and induce leakage and ultimate lysis of eukaryotic cells. The haemolysin-α, also known as α-toxin, is secreted as a monomer and associates as a homo-multimeric pore in the membrane of the host target cells [95]. Although it is present in all clinical isolates, recent studies have clearly demonstrated a primary role for α-toxin in experimental CA-MRSA infections. CA-MRSA strains deficient for

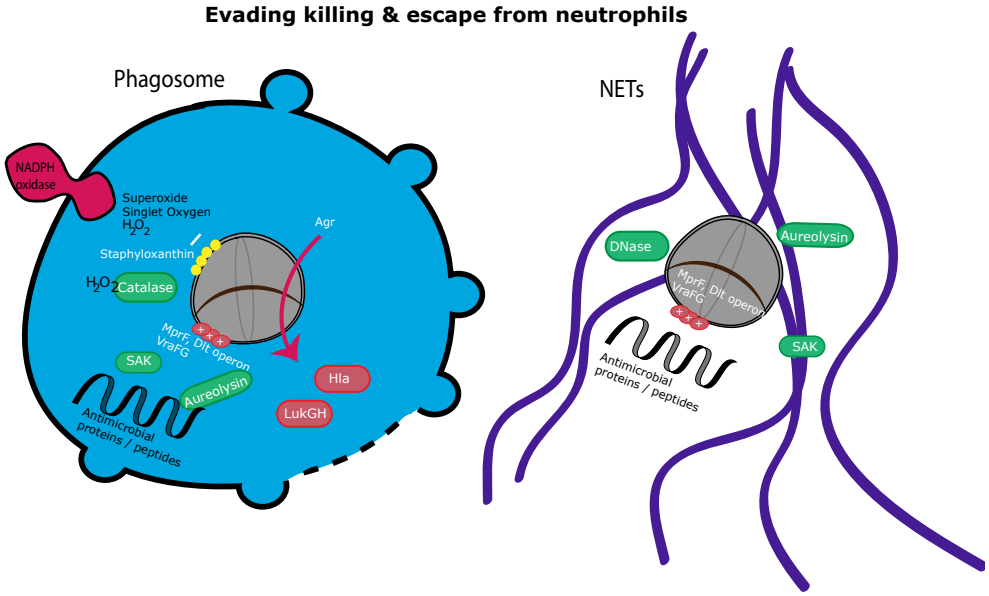

Figure 4. Evasion of neutrophil killing. Schematic representation of the mechanisms deployed by *S. aureus* avoiding killing inside neutrophils or by neutrophil extracellular traps NETs. Staphyloxanthin provides an antioxidant shield, whereas catalase detoxifies hydrogen peroxide. Resistance to cationic antimicrobial peptides (AMPs) is mediated by positive charge modifications of the cell wall, aureolysin-mediated proteolysis, and binding/inactivation by staphylokinase (SAK). Induction of the stringent response and quorum sensing mechanism agr leads to intraphagosomal production of Phenol soluble modulins (PSMs), LukGH and Haemolysin A (Hla) and thereby lysis of the membranes. Staphylococcal DNase can cleave the DNA backbone of NETs.

α-toxin were avirulent in a murine pneumonia model of infection compared with isogenic wild-type strains. Active and passive immunization against α-toxin protected animals from death in the same model [96,97]. Even though α-toxin is not cytotoxic to neutrophils, it lyses other immune cells like macrophages, lymphocytic subpopulations and erythrocytes [98]. In addition, it targets non-immune cells like epithelial cells. This target cell specificity suggests the involvement of a receptor. Recently, α-toxin was published to bind and upregulate the epithelial zinc-dependent metalloprotease ADAM-10 [99]. Although usage of this receptor offers an explanation for observed features of acute lung injury during staphylococcal pneumonia, it does not explain the leukocidal effects of this toxin.

The bi-component β-barrel pore-forming leukocidins comprise two subunits which are independently secreted as monomers and form hetero-multimeric pores in the membrane of host target cells [100,101,102]. Until now, five leukocidins of *S. aureus* have been described: Panton-Valentine Leukocidin (PVL), haemolysin-γ (Hlg), Leukocidin E/D (LukED), Leukocidin G/H (LukGH), and Leukocidin M/F-like (LukMF). LukMF has been associated to *S. aureus* strains causing mastitis in cattle, but has never been found in human isolates [103,104].

Hlg lyses both erythrocytes and leukocytes [105,106,107], while the other leukocidins are stricktly toxic to leukocytes [108,109,110,111]. Although the hlg and lukDE genes are present in the chromosome of 99% of all *S. aureus* isolates, the genes encoding pvl are located on bacterial pro-phages and are found only in 2% of all isolates [112]. In contrast to methicillin-susceptible *S. aureus* and hospital-acquired (HA-) MRSA, the majority of CA-MRSA isolates carry the pvl genes [5,113]. PVL is associated with severe invasive disease in humans, especially necrotizing pneumonia and osteomyelitis [114,115]. Its role in deep-seated primary skin and soft tissue infections is questionable. Large epidemiological studies have indicated that, at least for skin and soft tissue infections, PVL does not seem to determine clinical outcomes [116,117]. Experimental confirmation of the contribution of PVL to pathogenesis of *S. aureus* has resulted in an intense international debate [96,118,119,120,121], since mice are fully resistant to this cytolytic toxin. Further investigations revealed a clear species specificity of PVL cytotoxicity, where mice and macaque neutrophils were resistant, but human and rabbit neutrophils susceptible [122]. The species specificity of these bi-component toxins most likely is determined by the interaction with host GPCRs, as was demonstrated recently for LukED [123]. By targeting GPCRs, *S. aureus* has a highly selective tool to attack phagocytes before the pathogen is engulfed.

Phenol soluble modulins (PSMs)

PSMs were first described as pro-inflammatory components in phenol-extracted *S. epidermidis* culture filtrates, hence their name: phenol soluble modulins. Three small hydrophobic peptides, including the long-known δ-toxin were identified, which in culture supernatants formed a poly-peptide complex [124]. Almost ten years later, the group of Micheal Otto published the landmark paper in Nature Medicine, describing PSMs as key virulence factors of *S. aureus* [30]. Since then the group has been really productive in attributing a variety of biological functions, crucial to staphylococcal pathogenesis, to PSMs.

PSMs are unique for the staphylococcus genus. Next to *S. epidermidis* and *S. aureus*, several other staphylococcal species express PSMs, [125,126] however, PSMs have not been found in other bacteria. Staphylococci, particularly pathogenic species such as *S. aureus*, secrete PSMs in extremely high amounts; making them by far the most abundant proteins in staphylococcal culture filtrates [30]. All PSMs share a common amphipathic α-helical structure and are categorized in two groups depending on their size: the smaller α-type (20-30 amino acids) and the larger β-type (44 amino acids) [30] (**Figure 5**). Some PSMs, such as the PSMα group of *S. aureus* or the PSMα peptide of *S. epidermidis*, facilitate lysis of neutrophils, peripheral blood mononuclear cells, and erythrocytes. Why β-type PSMs lack such cytolytic activities is still unclear [30,127]. As a consequence of their lytic properties, expression levels of PSMs seem to determine the strain-to-strain difference in virulence [30,128]. Furthermore, genetic deletion mutants of the *psm*α operon of two CA-MRSA strains had a strong impact on neutrophil recruitment and lysis *in vitro* and *in vivo*, and on the outcome of skin infection and bacteremia, highlighting a prominent role for α-type PSMs in CA-MRSA disease [30]. PSMs are not species specific, as reduced virulence for *psm*α mutants was shown in experimental mice and rabbit disease models [30,129]. All PSMs are located on the core genome and the sequence is highly conserved, except for PSM-*mec*, which is located on the methicillin resistance-encoding MGE staphylococcal cassette chromosome SCC*mec* [130]. The production of PSMs is strictly controlled by the staphylococcal quorum-

sensing mechanism accessory gene regulator (*agr*). This system controls the expression of virulence determinants such as lipase, protease and toxins, in a cell-density dependent manner. The *agr* system utilizes the peptide pheromone auto inducing peptide (AIP), which, when accumulated to a certain threshold concentration, binds to trans-membrane histidine kinase (AgrC). AgrC phosphorylates and subsequently activates the response regulator protein; AgrA. In turn, AgrA binds and induces transcription of two promoters present in the *agr* system First, it induces transcription of the entire *agr* operon (positive feedback loop). Secondly, AgrA upregulates the expression of the intercellular effector molecule RNA III. While RNA III is responsible for the increased production of proteases, lipases, leukocidins, α-toxin and enterotoxins, PSMs are directly induced by AgrA binding to their promoter [131]. Next to their lytic activities, all PSMs trigger inflammatory responses via the formyl peptide receptor (FPR) 2. Activation of this receptor leads to chemotaxis, priming of human neutrophils and induction of cytokine expression [31,125,126].

Another biological function of PSMs lies in the structuring and dissemination of biofilm-associated infections. Biofilms are surface attached bacterial agglomerations and are embedded in an extracellular matrix. Biofilm formation offers significant advantages for bacteria, as it protects against host AMPs and provides resistance against most antibiotics. Staphylococci are the most frequent cause of nosocomial biofilm-associated infections on indwelling medical devices, such as catheter, synthetic heart valves or bone prosthesis. PSMs disrupt the tight cellular interactions and mediate the dissemination from the sticky biofilms, in both *S. aureus* and *S. epidermidis* [132,133]. In addition, PSMs promote movement of staphylococci over wet surfaces [134,135].

Finally, PSMs might function as weapons against other bacteria and thereby create a niche for staphylococcal colonization. Human antimicrobial peptide LL-37 has high

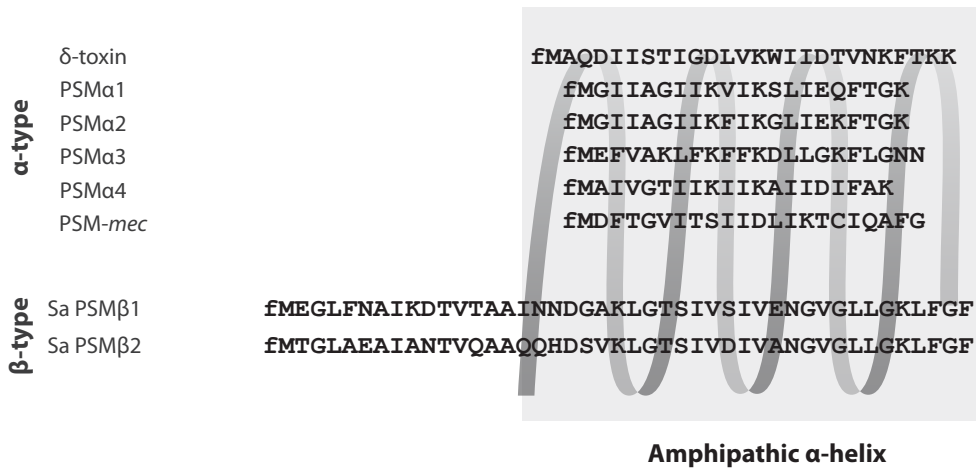

Figure 5. Phenol-soluble modulins (PSMs) present in *S. aureus.* PSMs are a family of core genome–encoded peptides (except PSM-*mec*) with surfactant properties secreted by staphylococci. PSMs usually do not share significant amino acid sequence similarity, but all contain an amphipathic α-helix important for surfactant properties.

structural resemblance with PSMs. Therefore, it is not surprising that some PSMs also have antimicrobial properties [136,137] or work in cooperation with host antimicrobial peptides [138]. For instance, PSM derivatives can efficiently kill other Gram-positive bacteria such as *Streptococcus pyogenes*.

Escape from neutrophils

Multiple lines of evidence have accumulated recent years that *S. aureus* can survive, or even use host cells as transport to disseminate from the site of infection [139]. Especially CA-MRSA strains have enhanced capacity to lyse neutrophils after phagocytosis leading to increased bacterial survival [140]. It has recently been described that the concentration of AIP, responsible for gene transcription activation of the staphylococcal quorum sensing mechanism *agr*, can reach the critical concentration within cells. This allows the *agr* system to function within the phagosome of monocytes [141], resulting in escape in an α-toxin dependent manner. However, the importance of single virulence factors contributing to intracellular survival seems to be dependent on the type of host cell and bacterial strains analysed. For instance, in the USA 300 strain LAC, LukGH may have a role in intracellular neutrophil lysis [142].

Aim of this thesis

The innate immune system is crucial in destroying invading bacteria. However, pathogenic *S. aureus* strains have evolved numerous mechanisms to circumvent their killing. In this study, we challenge the current hypothesis that PSMs function as extracellular toxins. Furthermore, we hypothesize that another pathogen, *Streptococcus pneumoniae*, in a similar fashion as *S. aureus*, secretes immune evasion molecules.

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2

Innate Recognition of Bacteria

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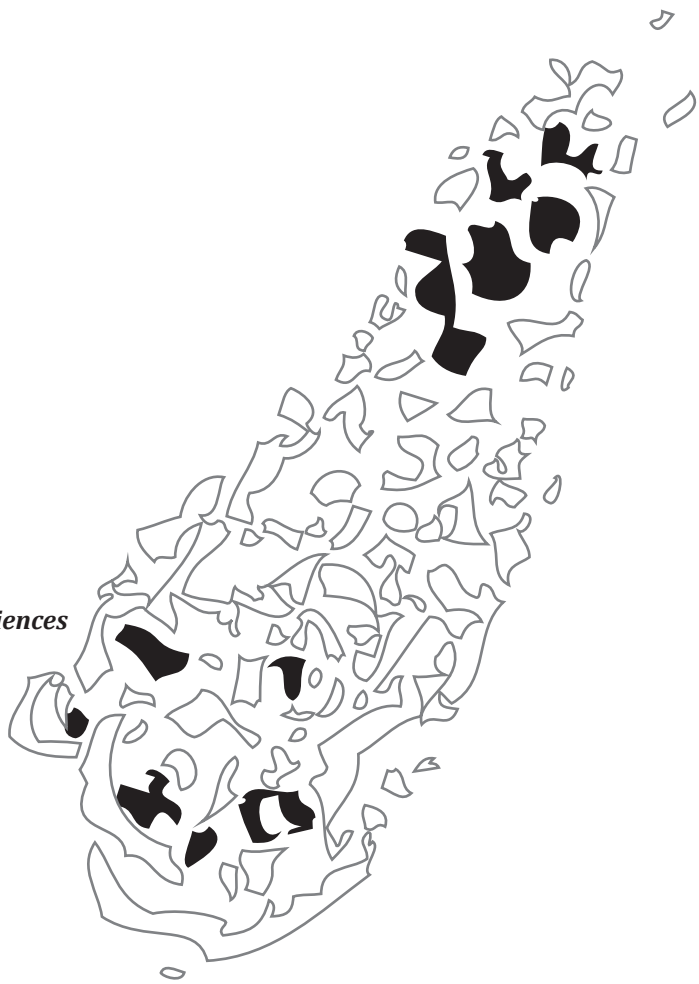

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Abstract

Innate immunity is the first line of defense against bacteria and other microbes and has evolved to eliminate invading bacteria as quickly as possible. Molecular recognition is a key element in this process, how does our innate immune system distinguish self from non-self? And how can it recognize all bacteria? It has been estimated that there are over a million species of bacteria! The answer is simple; to recognize all bacteria our innate immune system recognizes evolutionary conserved structures. In this overview we will, step by step describe groups of receptors and their subsequent ligands. We will illustrate most examples both at the functional as well as the molecular level.

Introduction

Our innate immune system is not solely designed to eliminate pathogens, the primary task is the fast an efficient elimination of all invading microorganisms. However, those bacteria that escape from the attack of the innate immune system can become pathogens. Therefore innate recognition involves sensing of over a million species of bacteria. Adaptive immunity, involving T cell recognition and antibodies produced by B-cells, is acquired during life. The innate immune system is present in our genome and not adapted during life, with less than 30000 genes in a human genome this recognition of so many bacteria seems an impossible task. In order to recognize all these microbes our innate immune system senses evolutionary conserved structures. These highly conserved molecular signatures are present in bacteria and not in our own body.

Innate immunity

Our innate immune system consists of three important effector mechanisms that rapidly and efficiently destroy microbes; antimicrobial peptides, the complement system and professional phagocytes.

Antimicrobial peptides such as LL37, cathelicidins, cathepsins and α-defensin, directly insert into the microbial membrane, thereby lysing the microbe within seconds to minutes. However, these peptides have to reach high concentrations to be effective. Within the gut, on the skin or inside phagocytes (phagosome) these high concentrations can be reached [1].

The complement system consists of 30 proteins, all present within the human blood. Activation triggers a proteolytic cascade leading to several antimicrobial effector functions. Uncontrolled complement deposition has detrimental effects for the host, so activation is tightly regulated. Three separate pathways that differ in their mode of recognition initiate activation, but all converge at one central step: the cleavage of complement component C3. C3 cleavage results in the deposition of C3b molecules on the microbial surface and marks the microbe for efficient removal by phagocytes (opsonization). Further downstream the complement cascade splits C5 into C5a and C5b. C5a is a very potent chemoattractant for phagocytes and C5b is the first component of the C5b-9 complex, a membrane attack complex that can lyse Gram-negative bacteria [2].

Professional phagocytes (such as neutrophils, monocytes and macrophages) can ingest and kill bacteria within a minute. Of these cells, neutrophils are most abundant (50% of all white blood cells in humans) and are best equipped to fight bacteria. Upon infection blood borne neutrophils emigrate from the vessel lumen through the lining endothelium into the underlying tissue. Inflammatory mediators and chemokines released by host cells activate endothelium cells locally, which in turn up-regulate specific receptors to guide neutrophils to the site of infection. The newly arrived phagocytes immediately recognize hostile bacteria, either directly by surface receptors that recognize structurally conserved bacterial molecules, by complement receptors that recognize a coating of complement proteins, or by Fc-receptors that recognize antibodies on the bacterial surface. Activation of complement receptors or Fc-receptors, which recognize the constant fragment of immunoglobulins, initiates phagocytosis, phagosome formation and bacterial killing. Phagocytosis is accompanied by the formation of the NADPH oxidase complex, which

generates the highly reactive oxygen species (ROS) and fusion of cytoplasmic granules with microbe-containing phagosomes (degranulation). Degranulation enriches the phagosome lumen with antimicrobial peptides and proteases, which in combination with ROS create a detrimental environment to kill any ingested microbe [3].

Bacterial structure and potential evolutionary conserved structures

A schematic representation of the cell wall structure from Gram-negative and -positive is depicted in **Figure 1.** Gram-negative bacteria are characterized by two lipid bilayer membranes with a periplasmic space in between. Peptidoglycan is present in the periplasm and provides structure and rigidity for the bacterium. Lipopolysaccharide (LPS) is an important constituent of the outer leaflet of the outer membrane, providing support and membrane stability.

Gram-positive bacteria have a considerable thicker peptidoglycan layer than Gram-

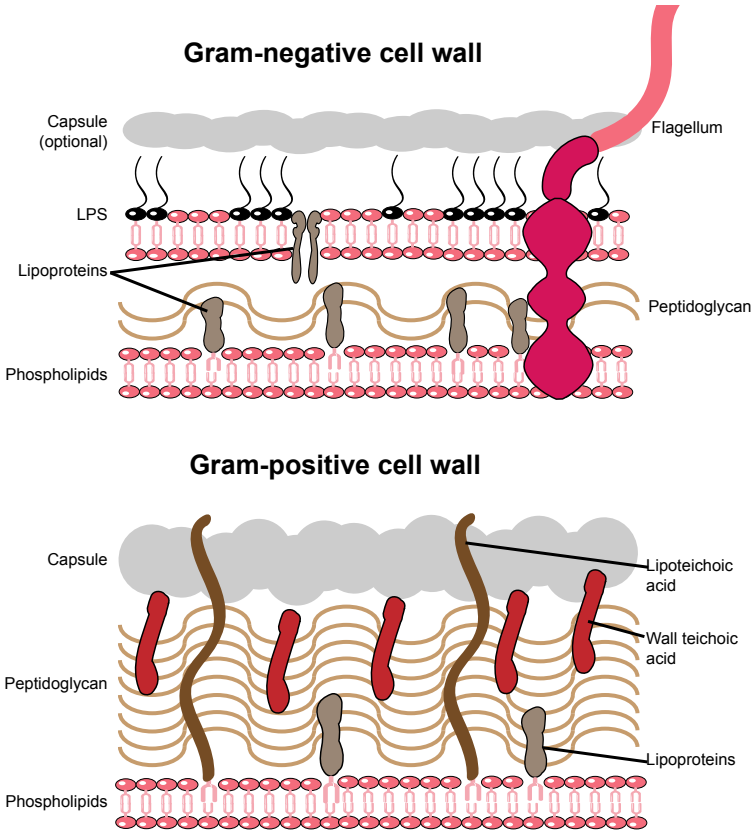

Figure 1. Schematic representation of cell wall composition of Gram-negative and -positive bacteria. Gram-negative bacteria have an outer membrane that contains lipopolysaccharide (LPS), furthermore a thin peptidoglycan layer is located between the two membranes. Gram-positive bacteria are characterized by a thick peptidoglycan layer and teichoic acids in their cell wall. Both Gram-positive and -negative bacteria can contain a capsule and/or flagella.

negatives, furthermore they do not have an outer membrane. Many conserved structures of the bacterial cell wall are distinct from eukaryotic structures and are therefore the perfect targets for distinguishing self from non-self by eukaryotic innate immune receptors. Specific cell wall components as peptidoglycan and teichoic acids are obvious targets for the innate immune system, but also flagellin, LPS and lipoproteins serve as targets for the innate immune system.

Toll Like Receptors (TLRs), formyl peptide receptors (FPR) and a wide variety of lectins and lectin-like molecules can recognize these conserved bacterial molecules. Here we will give an overview of the most important innate immune receptors involved in bacterial recognition.

TLRs

The family of TLRs recognizes a wide variety of conserved microbial structures. These receptors are characterized by an extracellular leucine rich repeat (LRR) domain, a trans membrane domain, and an intracellular Toll/interleukin 1 signaling domain. TLRs are expressed on various cells of the immune system and can be found on the plasma membrane, but also intracellular in endosomal compartments. Activation of TLRs triggers the recruitment of intracellular adapter proteins, which initiate a signaling cascade leading to production of inflammatory cytokines and recruitment of various cells to the site of infection. Structural studies between receptor and ligand have provided insight in recognition of several conserved bacterial ligands and activation mechanism of TLRs [4], we will discuss the ligand recognition of different TLRs below (**Figure 2).**

TLR4-LPS

TLR4 recognizes lipopolysaccharide (LPS), which is the major component of the outer leaflet of the outer membrane of Gram-negative bacteria. LPS consists of a hydrophobic lipid A domain, an oligosaccharide core and a distal polysaccharide domain (O-antigen). Lipid A anchors LPS to the outer membrane of Gram-negative bacteria and triggers a strong innate immune response via TLR4, which can cause septic shock. Assistance of LPS-binding protein (LBP) and CD14 is required to realize efficient recognition of LPS. LBP solubilizes and transfers LPS to the membrane anchored CD14, which presents LPS to TLR4 in complex with MD-2 (Fig. 2). This receptor complex triggers inflammatory intracellular signaling pathways.

The structure and synthetic pathway of lipid A is conserved in Gram-negative bacteria, in contrast to the surface exposed O-antigen domain. LPS is crucial for stabilization and fitness of Gram-negative bacteria and therefore lipid A is an ideal target of the innate immune system. Lipid A consists of two glucosamines, which can be phosphorylated, connected to a varying number of acyl side chains (4-7) of slightly different lengths. The biological activity of LPS depends on the number of acyl chains [5], deletion or addition of an acyl chain reduces activity up to 100-fold. Some bacteria manipulate the number of acyl chains of their lipid A structure under certain circumstances to impair the recognition by TLR4 and thereby reduce the inflammatory response [6].

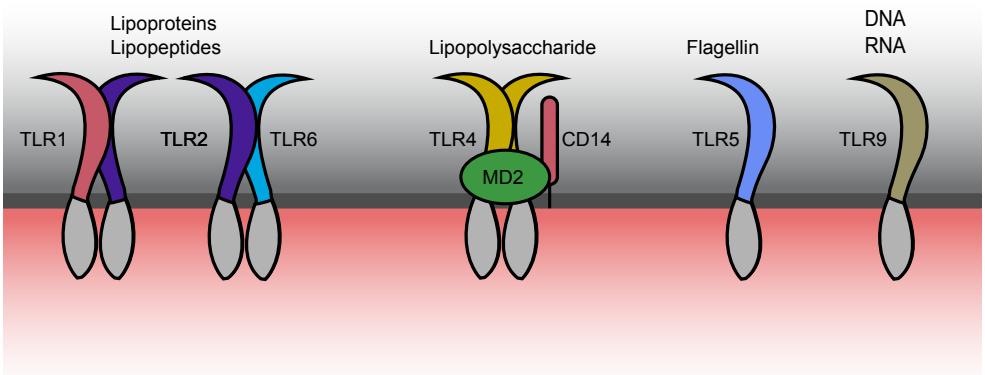

Figure 2. Simplified overview of the TLR family.
TLRs are expressed on various cells of the immune system and recognize a wide variety of ligands of different origin. They form either homo- or heterodimers and activate downstream signaling pathways via their intracellular domain, leading to production of pro-inlammatory cytokines and activation.

TLR2 - lipoproteins

The list of described TLR2 ligands contains a wide variety of structurally diverse molecules [7]. The most potent activators of TLR2 are bacterial lipoproteins that contain a specific modification of the N-terminal cysteine residue. This modification is unique to bacteria and the enzymes in lipoproteins biogenesis are strongly conserved in bacteria. In general, Gram-negative bacteria produce proteins with a triacylated N-terminal cysteine, whereas lipoproteins of Gram-positive bacteria are diacylated. The conserved lipid modification anchors even highly hydrophilic proteins to the hydrophobic bacterial membrane. Lipoproteins have several important functions in bacteria such as membrane stabilization, drug efflux, and are involved in virulence. Other described ligands activate TLR2 only at higher concentrations and is questionable if these are physiological relevant concentrations. Experiments with *Staphylococcus aureus* illustrate that lipoproteins are the major ligands for TLR2. Mutants that lack maturated lipoproteins fail to activate TLR2, despite the presence of described TLR2 ligands such as lipoteichoic acid and peptidoglycan [8].

TLR2 cooperates with TLR1 and TLR6 to increase ligand specificity by forming heterodimers. TLR2 in combination with TLR1 recognizes di- and triacylated lipoproteins, whereas the TLR2/6 heterodimer recognizes only triacylated lipoproteins. Recognition depends not only on the number of acyl chains, but also on the sequence of the peptide connected to the modified cysteine. Recent structural studies provide insight in ligand recognition and discrimination between di- and triacylated lipopeptides by TLR1, 2, and 6 [4].

TLR5 - flagellin

Several bacteria use flagella to swim towards chemotactic or thermotactic gradients. Flagella are required for colonization and virulence of pathogenic bacteria. Bacteria swim to their target by counterclockwise rotation (run) and by clockwise rotation of the flagellum (tumble) they can change their swimming direction. The bacterial flagellum consists of a

basal body (motor), a hook structure, and a long filament composed of up to 20,000 flagellin molecules. Flagellin consists of different domains that dramatically differ in conservation between bacterial species. The D0-D1 domain interacts with adjacent flagellin monomers and is highly conserved, whereas the D2-D3 that sticks outside the flagellum is highly variable.

TLR5 recognizes an epitope in the highly conserved D0-D1 domain of flagellin and is the only TLR that recognizes a single ligand of protein origin. Since, this domain is normally not accessible in the native state (flagella) only monomeric flagellin is recognized by TLR5 [9]. The surrounding of bacteria contains monomeric flagellin, probably due to some degradation of flagella. Since, a single flagellum contains 20,000 flagellin monomers and TLR5 recognizes flagellin in the picomolar range disintegration of some flagella is sufficient for proper TLR5 activation. Mutations in the TLR5 recognition domain of flagellin result not only in the loss of TLR5 activation, but also in loss of motility. Modification of flagellin has severe fitness costs for the bacteria and is therefore an ideal target to be sensed by the innate immune system. Some bacteria, like Helicobacter pylori and Campylobacter jejuni, managed to modify their flagellin in such a way that TLR5 activating properties are lost, whereas motility is maintained.

TLR9-bacterial DNA

Sensing microbial DNA sounds as a hard challenge, since this molecule is present in all living organisms. The innate immune system manages to discriminate between bacterial and self DNA by recognizing hypomethylated CpG-dinucleotides present in bacterial DNA. Mammalian DNA contains a lower frequency of CpG dinucleotides, and these are mostly not methylated. In contrast to plasma membrane localized TLRs described above, TLR9 resides in the endosomal membrane of B cells and plasmocytoid dendritic cells. However, many other cells that lack TLR9 also respond to microbial DNA. This initiated new efforts to identify other sensors of the innate immune system resulting in the identification of at least six other intracellular receptors involved in sensing microbial DNA [10]. The patterns recognized by these sensors are still unknown, and for some it has been shown that they cannot discriminate between microbial and mammalian DNA.

Inflammasome

Several bacterial molecules can activate intracellular multiprotein complexes called inflammasomes. Activation leads to cell death via pyroptosis and results in maturation of proinflammatory cytokines IL-1beta and IL-18. Until now, four inflammasomes have been described of which three contain a member of the Nod-like receptor (NLR) family that regulates their activity (NLRP1, NLRP3, NLRC4). Upon ligand recognition NLRs oligomerize and recruit other proteins to form the multiprotein complex that is called the inflammasome.

The NLRC4 inflammasome recognizes bacterial flagellin and components of the type III secretion system (TTSS) of bacteria. For recognition of these various compounds different members of the neuronal apoptosis inhibitor protein (Naip) family are required. Naip2 plays a role in recognizing PrgJ-like proteins, that form the basal body rod component of the TTSS secretion system. Naip5 interacts with flagellins of different bacteria, but does not recognize all different flagellins. Both Naips interact with similar structures that lie within

the C-terminal domain of both flagellin and TTSS rod shaped proteins [11].

Various bacteria have been described to activate the NLRP3 inflammasome, but for most of them the ligand is still unkown. Bacterial toxins like streptolysin O and cholera toxin activate the NLRP3 inflammasome, since these toxins have very different mechanisms of action it is still unclear how they activate NLRP3.

GPCRs

G-protein coupled receptors (GPCRs) form a large family of receptors that can bind a variety of ligands such as light-sensitive compounds, odors, pheromones, hormones, and neurotransmitters. However, in immunology the GPCRs sensitive to chemoattractants are the most studied receptors. Neutrophils sense gradients of chemotactic factors via GPCRs to navigate to the site of infection. Well-known host derived chemoattractants for phagocytes are platelet activating factor PAF, leukotriene B4, C5a and interleukin-8 (IL-8). Next to host-derived chemoattractants, bacterial products, such as fMLP, can also serve as potent inducers of GPCR signaling. Bacteria synthesize proteins in a different way than eukaryotes and archaea and therefore certain host GPCRs can distinguish self from non-self in this process. During protein synthesis translation starts with a methionine residue that can be removed during protein maturation. While archaeic and eukaryotic proteins are synthesized with an unmodified primairy methionine residue; newly synthesized bacterial proteins contain a formylated methionine at their N-terminal end. Therefore growing bacteria are

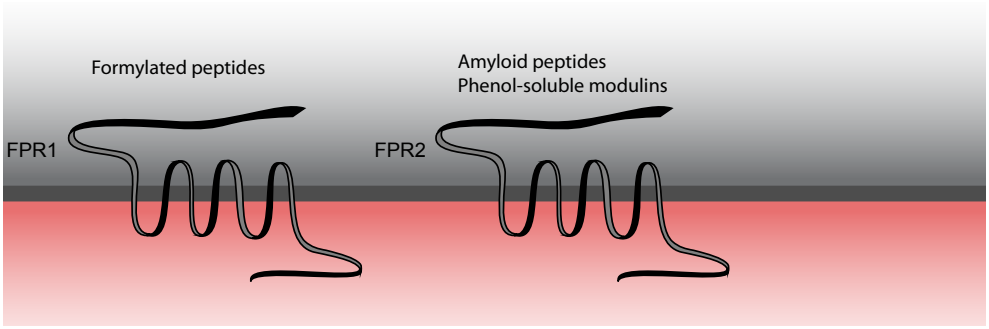

Figure 3. Schematic representation GPCRs involve in sensing bacterial ligands. Formylated peptide receptor (FPR) 1 and 2 play a key role in innate immunity by sensing formylated peptides or phenol-soluble modulins produced by bacteria, activation of these receptors leads to priming, activation and chemoattraction.

surrounded by a substantial gradient of N-formylated proteins and peptides. The prototype N-formyl-peptide; fMLP, induces chemotaxis, phagocytosis, and the generation of reactive oxygen species in both neutrophils and monocytes. Formylated peptides act on receptors belonging to formylated peptide receptor (FPR) family **Figure 3**. FPR1 binds fMLP with high affinity, while FRP2 (also known as FPRL-1) has low affinity for this ligand. Both FPR1 and FPR2 are expressed on neutrophils, whereas monocytes have an additional orphan FPR3.

Bacteria are not the only source of formylated peptides; mitochondria of eukaryotic cells, since they are of bacterial origin, also use N-formylmethionine as initiator of protein synthesis. When cells die of necrosis, danger signals in the form of formylated peptides are

released and are a major cause of inflammatory reactions [12].

Staphylococcus aureus actively secretes vast amounts of small cytolytic peptides, called phenol-soluble modulins (PSMs) [13]. These PSMs are N-formylated and have the ability to activate and attract neutrophils at nanomolar concentrations and be cytolytic at micromolar concentrations. Enhanced expression of PSMs that can be found in community-acquired methicillin-resistant *S. aureus* (CA-MRSA) contributes to its enhanced virulence. FPR2, which was known to sense endogenous inflammatory amyloidogenic ligands, senses PSMs at nanomolar concentrations and initiates proinflammatory neutrophil responses to Staphylococci [14]. Recently, Schwartz and co-workers [15] showed that PSMs can form amyloid fibres. Taken together with the information on human ligands for FPR2, which are all amyloid structures it is tempting to speculate that FPR2 is a pattern recognition receptor that senses amyloid structures from either microbial or mammalian origin.

Lectins

Cell wall-associated lectins

A whole array of different cellular receptors and soluble lectins or lectin-like, interact with carbohydrates and carbohydrate motives, which facilitates the discrimination between non-self and self as illustrated in **Figure 4.**

Membrane-associated lectins receptors recognize and differentiate between the glycans of foreign microbes and endogenous ligands. Only sensing of microbial glycans initiates an inflammatory response. C-type lectins that are cell bound include the mannose receptor, dectin-1 and DC-sign. On macrophages DC-sign and the mannose receptor play a role in

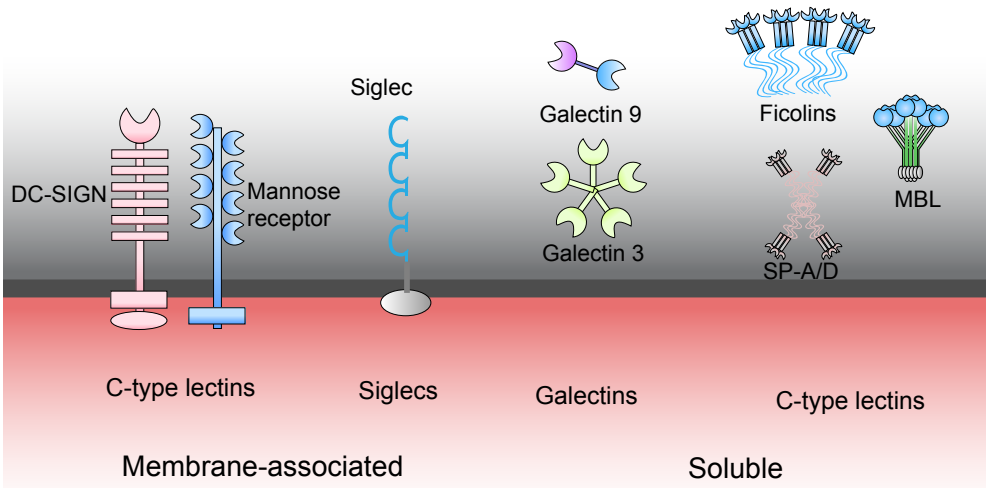

Figure 4. Overview of carbohydrate recognition molecules of the innate immune system. The family of C-type lectins consists of receptors like DC-SIGN involved in cellular activation and of soluble pattern-recognition molecules such as mannose binding lectin that can activate the complement system. Siglecs can recognize sialylated glycoconjugates and can trigger cellular activation. Galectins bind to N-acetyllactosamine-containing sugars on bacterial surfaces.

immune recognition of mannosylated carbohydrates and sulfated ligands of bacteria, fungi and viruses [16]. Whereas all other lectins can only provide inflammatory signals, Dectin-1 can mediate phagocytosis. Siglecs (sialic acid binding Ig-like lectins) recognize sialylated glycoconjugates and are characterized by an N-terminal Ig domain. They are present on a variety of white blood cells (macrophages, B-cells) and play a clear role in cellular activation as well as deactivation [17].

Soluble lectins

The collagenous lectins or collectins (e.g. mannose-binding lectin (MBL), surfactant proteins SP-A and SP-D and conglutinin) and the ficolins are soluble pattern-recognition receptors. They are present in human plasma or in the lung act as indirect recognition molecules. Some activate the complement pathway (MBL and the ficolins), others interact with cellular receptors to provoke inflammatory events. MBL recognizes different bacteria [18] and is important in innate recognition. Ficolins are a family of oligomeric proteins consisting of an N-terminal collagen-like domain and a C-terminal globular fibrinogen-like domain. Ficolins specifically recognize N-acetyl compounds such as N-acetylglucosamine, components of bacterial and fungal cell walls, and certain bacteria. Just as MBL, ficolins are in complex with MBL-associated serine proteases (MASPs). These MASPs activate the complement through the lectin pathway. This leads to phagocytosis and lysis of bacteria. In humans, three ficolins (L-, M- and H-ficolins) have been identified, which exhibit differences in ligand binding and bacterial recognition, suggesting specific roles for each ficolin.

Specificity of these lectins for pathogens is determined by density-dependent glycan binding and pattern-recognition and not by the nature of the monosaccharides involved. In many cases, these monosaccharides form a low affinity epitope. Host lectins may bind to such “weak epitopes” due to large increases in the affinity of the lectin for polyvalent displays of the glycan. Two mechanisms are identified for lectins binding to density dependent glycans: (1) binding and internal diffusion of lectins such as galectins among a dense population of glycans, and (2) binding of multiple CRD domains of lectins such as soluble C-type lectins to clustered glycan epitopes or a combination of both.

Summary and perspective

By recognizing evolutionary conserved bacterial structures immune cells can distinguish self from non-self and recognize a vast variety of bacteria and microbes in general. In this way our immune cells are alerted by using only a limited number of innate recognition molecules, which can recognize all bacteria, viruses and fungi. However, bacteria and viruses have co-evolved with host cells and developed various mechanisms to evade recognition by the innate immune system, and thereby escape killing mechanisms to become a pathogen. In the past decade, a wide variety of bacterial immune evasion molecules have been identified and characterized. Especially, *S. aureus* seems to be well equipped by having an arsenal of over twenty-five toxins and immune evasion molecules [3,19]. These immune modulators disarm various branches of the innate immune system by blocking crucial steps in recognition and killing mechanisms. In future vaccine development strategies it would be very beneficial to counteract especially these immune evasion strategies. This will, especially locally, restore the normal functioning of our own highly efficient immune system and in this way, clear bacterial infections.

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3

Inactivation of Staphylococcal Phenol-Soluble Modulins by Serum Lipoprotein Particles

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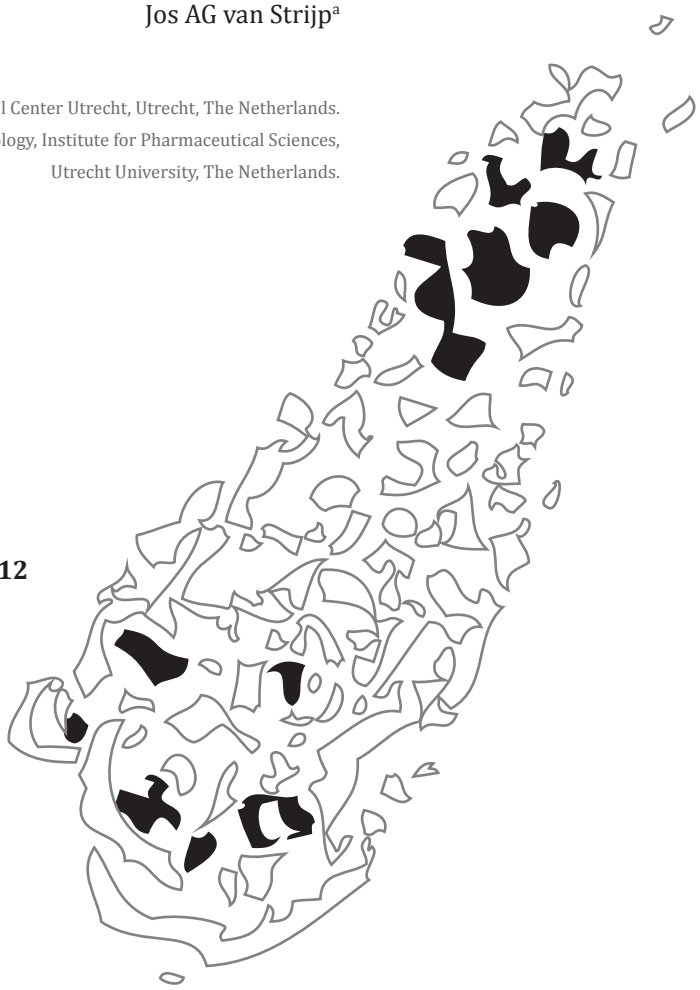

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Abstract

Staphylococcus aureus virulence has been associated with the production of phenol-soluble modulins (PSMs). PSMs are known to activate, attract and lyse neutrophils. However, the functional characterizations were generally performed in the absence of human serum. Here, we demonstrate that human serum can inhibit all the previously-described activities of PSMs. We observed that serum can fully block both the cell lysis and FPR2 activation of neutrophils. We show a direct interaction between PSMs and serum lipoproteins in human serum and whole blood. Subsequent analysis using purified high, low, and very low density lipoproteins (HDL, LDL, and VLDL) revealed that they indeed neutralize PSMs. The lipoprotein HDL showed highest binding and antagonizing capacity for PSMs. Furthermore, we show potential intracellular production of PSMs by *S. aureus* upon phagocytosis by neutrophils, which opens a new area for exploration of the intracellular lytic capacity of PSMs. Collectively, our data show that in a serum environment the function of PSMs as important extracellular toxins should be reconsidered.

Author Summary

Infections with methicillin-resistant *Staphylococcus aureus* (MRSA) are difficult to treat because of resistance against standard antibiotics. In contrast to the traditional hospital-associated (HA-) MRSA strains, community-associated (CA-) MRSA strains cause severe infections in otherwise healthy individuals. CA-MRSA strains display enhanced virulence, spreading more rapidly and causing more severe illness than HA-MRSA strains. Enhanced virulence of CA-MRSA is thought to be associated with the production of several toxins, such as Phenol-Soluble Modulins (PSMs). PSMs have been described to activate, attract and lyse neutrophils. Thus far, previous studies characterizing the functions of PSMs were performed in the absence of body fluids. In the current study, we show that human serum strongly inhibits many functions attributed to PSMs. We demonstrate that serum lipoprotein particles are responsible for the binding and inhibition of PSMs, even when PSMs are produced by growing *S. aureus* in whole blood. Finally, we show production of PSMs by *S. aureus* within neutrophils, suggesting that PSMs may play a role intracellularly in a serum-free environment. These findings significantly contribute to our understanding of the function of PSMs and strongly suggest that PSMs, instead of performing as extracellular toxins, most likely act as intracellular toxins.

Introduction

Staphylococcus aureus frequently colonizes human anterior nares and can cause many infectious diseases, ranging from mild superficial skin and wound infections to life-threatening disseminated infections [1]. The number of infections by this bacterium is increasing, especially infections caused by methicillin-resistant *S. aureus* (MRSA) strains. However, infections are still limited to a small percentage of colonized individuals. This suggests that the human innate immune system together with physical and humoral barriers can very effectively control invasive infections, even those caused by the invasive community-associated (CA) MRSA. Therefore, we hypothesize that virulence factors produced by *S. aureus* are likely generally counteracted by the innate immune system, and that a balance between the two determines the outcome of an infection.

In order to survive within the host, *S. aureus* can make use of a variety of virulence factors [2], including a repertoire of toxins [3]. The toxins induce host cell lysis and include superantigens, leukocidins and phenol soluble modulins (PSMs). In contrast to most other toxins, PSMs are small core genome-encoded peptide toxins, except for PSM-*mec*, which is located on the methicillin resistance-encoding MGE staphylococcal cassette chromosome SCC*mec* [4]. The production of PSMs is controlled by the quorum-sensing accessory gene regulator (*agr*) [5], and the gene expression levels correlate with strain virulence. Especially CA-MRSA strains are associated with high productions of PSMs, which is thought to account for the enhanced virulence, easier spreading and severity of infection of CA-MRSA strains compared to hospital-acquired MRSA strains (HA-MRSA) [6], [7]. Thus far, all described PSMs have a common amphipathic alpha helical region, which is thought to enable their cell lytic ability most likely by disrupting the cell membrane [7]. Despite having a similar structure, PSMs are categorized in two groups, depending on their size. The smaller α-type PSMs (PSMα1, PSMα2, PSMα3, PSMα4, and δ-toxin), with a length of 20–30 amino acids, are regarded as the most toxic PSMs [7], whereas the larger β-type PSMs (PSMβ1 and PSMβ2) of approximately 44 amino acids seem to have additional functions. For instance, β-type PSMs of S. *epidermidis* are described to play a role in biofilm dispersal [8].

Next to lysing neutrophils, PSMs are described to activate and attract leukocytes. Neutrophils are the first leukocytes recruited to the site of infection and are crucial in controlling staphylococcal infections. They are attracted by both host factors and conserved microbial molecules also known as pathogen-associated molecular patterns (PAMPs). Although many PAMPs are recognized by Toll-like receptors (TLRs) [9], PSMs are potent staphylococci-specific PAMPs which act mainly on the human formylated peptide receptor 2 (FPR2) [10]. FPR2 is expressed on neutrophils, monocytes, macrophages, immature dendritic cells, and microglial cells, and its activation induces many neutrophil effector functions, including chemotaxis, exocytosis and superoxide generation [11]. While micromolar concentrations of PSMs are needed for neutrophil lysis, nanomolar concentrations are enough for FPR2-mediated neutrophil stimulation. Although neutrophils sense PSMs at nanomolar concentrations, *S. aureus* can subvert FPR2 signaling by producing the antagonists FPR2 inhibitory protein (FLIPr) [12] and its homologue FLIPr-like [13].

S. aureus typically resides in mucosal and epithelial surfaces and can invade beyond these physical barriers causing invasive infections. The switch from a colonizing phenotype to a

virulent phenotype is regulated by *agr* [14]. This regulatory switch relies on the secretion of autoinducing peptide (AIP) sensed by the cell population and triggers the expression of virulence determinants such as proteases, haemolysins and toxins. To fight the infection, the human innate immune system has several effector mechanisms, both humoral and cellular, to clear the invading bacterium. Recently, Peterson *et al* [15] described a novel neutralizing mechanism; incorporating ApoB1 in VLDL and LDL lipoproteins in serum can sequester AIP and thereby disable staphylococcal quorum sensing. Mice lacking plasma ApoB1 are more susceptible to invasive staphylococcal infections, implicating that ApoB1 is an essential innate defense effector against *S. aureus* [15]. The current study shows that serum also provides a barrier against the pro-inflammatory and cytolytic activities of PSMs, the highest *agr*-upregulated virulence factor of S. aureus. Our main finding is that PSM-induced activation and lysis of neutrophils are greatly inhibited by human serum. Lipoprotein particles were identified as the PSM-binding and -inhibiting components within serum, suggesting that they function as scavengers of PSMs, thereby preventing host damage. We show that *S. aureus* potentially can produce PSMs inside neutrophils after phagocytosis.

Materials and Methods

Ethics statement

Informed written consent was obtained from all donors and was provided in accordance with the Declaration of Helsinki. Approval was obtained from the medical ethics committee of the University Medical Center Utrecht (Utrecht, The Netherlands).

Reagents

PSM peptides were synthesized with the recently published sequences [7] by Genscript at 95% purity. PSMα1 (MGIIAGIIKVIKSLIEQFTGK), PSMα2 (MGIIAGIIKFIKGLIEKFTGK), PSMα3 (MEFVAKLFKFFKDLLGKFLGNN), PSMα4 (MAIVGTIIKIIKAIIDIFAK), PSMβ1 (MEGLFNAIKDTVTAAINNDGAKLGTSIVSIVENGVGLLGK LFGF), PSMβ2 (MTGLAEAIANTVQAAQQHDSVKLGTSIVDIANGVGLLGKLFGF), δ-toxin (MAQDIISTISDLVKWIIDTVNKFTKK) were all synthesized with an N-terminal formyl methionine residue. Peptide stocks were prepared at 2 mM dissolved in H_2O except PSMα4, which was dissolved in 50% (v/v) MeOH/H_20. Peptide grade TFA, and HPLC grade MeOH were purchased from Biosolve. fMLP, FITC-isomers, and C5a were obtained from Sigma Aldrich. IL-8 was purchased from PeproTech. HDL, LDL, VLDL and ApoB1 were purchased from (Millipore). Apo-A1 was purchased from (Calbiochem). PVL components LukS and LukF were kindly provided by Gerard Lina, Centre National de Référence des Staphylocoques, Lyon, France.

Bacterial strains

In this study the *Staphylococcus aureus* strains COL, N315, MRSA252, Newman, MW2 and USA300 were used. MW2 *agr* knockout [35] was a kind gift of Alexander Horswill, the University Of Iowa, Iowa, USA. *S. aureus* strains were cultured overnight in Müller-Hinton broth (MHB) with shaking at 37°C. Alternatively, MW2 was grown in MHB until an OD_{660} of 1.0. Bacteria were washed with PBS and 10^6 CFU/ml was added to freshly drawn whole human blood anticoagulated with 50 μg/ml lepirudin (Refludan, Schering). Bacteria were cultured overnight in whole blood with shaking at 37°C. Bacterial culture supernatants or

whole blood culture plasma were clarified by centrifugation, filtered through 0.22 µm pore size filter and stored at −20°C in aliquots until use.

Human neutrophils

Human neutrophils were isolated by means of the Ficoll-Histopaque gradient method. Venous heparinized blood was diluted with an equal volume of PBS, and subsequently layered on a gradient of Ficoll (Amersham Biosciences) and Histopaque (Sigma Aldrich). After centrifugation for 20 min at 400 g and 21°C, polymorphonuclear cells (neutrophils) were collected from Ficoll and Histopaque interfaces. Cells were washed with cold RPMI-1640 containing 25 mM HEPES, L-glutamine (Biowhittaker), and 0,05% human serum albumin (HSA; Sanquin) (RPMI-HSA). Erythrocytes were lysed by applying a hypotonic shock to the neutrophil pellet with distilled H_2O for 30 sec, followed by 10× concentrated PBS to restore the isotonicity. The cells were washed and resuspended in RPMI-HSA. HL-60 cells stable transfected with the FPR2 (HL-60/FPR2), were kindly provided by F. Boulay (Laboratoire Biochimie et Biophysique des Systemes Integres, Grenoble, France). Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FCS), 2 µm, 100 units/ml penicillin, 100 µg/ml streptomycin, and 600 µg/ml G418.

Serum and lipoprotein preparations

Human pooled serum was obtained from at least 20 healthy donors and stored until use at −70°C. Serum was inactivated by heating at 56°C for 20 min. Isolation of lipid free serum was performed as described [1]. Briefly, EDTA-plasma or clarified lepuridin-plasma was applied on a gradient of potassium bromide and ultracentrifuged at 166.000 g for 22 h at 4°C. The lipid free serum fraction was isolated from the gradient with a density heavier than 1.25. The lipid fractions with a density between 1.063 and 1.210 and between 1.019 and 1.063 were used as the fractions containing HDL and LDL, respectively. Fractions were dialyzed against PBS, filtered (0.22 µm) and stored at 4°C until use. The concentration of HDL and LDL is expressed as the equivalent concentration of cholesterol in micrograms per milliliter. The purified lipoprotein and lipid free serum fractions obtained from the clarified lepirudin plasma were subject to a HPLC analysis. The lipid free serum fraction from the EDTA-plasma was subjected to a hexane extraction to remove the remaining lipid particles from the serum. The serum fraction and hexane 3:1 (v/v) were incubated for 30 min while vigorously shaking. The aqueous partition was dialyzed against PBS. The protein content was measured with a standard BCA kit and concentrated with an Amicon 10 kDa cut-off filter (Millipore) to adjust the protein content to the level present in normal human serum.

Calcium mobilization in human neutrophils and HL-60 cells

Calcium mobilization with isolated human neutrophils and HL-60/FPR2 cells was performed as previously described [13]. For this purpose, cells (5×10^6 cells/ml) were loaded with 2 µM Fluo-3-AM for 20 min at room temperature, protected from light with gentle agitation. The cells were washed, resuspended in RPMI-HSA (without FCS) to 5×10^6 cells/ml. Stimuli were prepared by incubating 25 µl of 10 times concentrated agonist with 25 µl 10 times concentrated heat inactivated serum, HDL, LDL or buffer for 30 min at room temperature. Before stimulation, cells were diluted to 1×106 cells/ml in a volume of 200 µl. The basal fluorescence level for Fluo-3 was monitored at 530 nm for 8 sec after which 50 µl of pre-incubated stimulus was added. The sample tube was rapidly placed back to the sample

holder and the fluorescence measurement continued up to 52 sec. Cells were gated based on scatter parameters to exclude cell debris and the mean fluorescence value at basal level was subtracted from the value at peak level (at 30 sec). The different fluorescent values were expressed as percentage of the maximal response for each individual stimulus. Alternatively, various concentrations of culture supernatants or synthetic PSMs, pre-incubated with 1% or 0,1% heat inactivated serum for 30 min, were added to Fluo-3-labeled HL-60/FPR2 cells followed by flow cytometry. For inhibition kinetics, 10 times concentrated PSMs were incubated with 0.1% serum and at different time-points of incubation samples were added as stimuli for HL-60/FPR2 cells in the flow cytometer.

Neutrophil lysis assay

Lysis of human neutrophils by filter-sterilized *S. aureus* culture supernatants or synthetic PSMs was measured as described [36], [37]. Clarified culture supernatants were pre-incubated with different concentrations of human serum for 10 min at room temperature. Pre-treated supernatants were transferred to a 96-wells ELISA plate (Nunc) containing 3×10^6 neutrophils in a total volume of 100 µl RPMI-HSA and were incubated for 15 min at 37°C. Neutrophil lysis was determined by release of lactate dehydrogenase (LDH) using the CytoTox 96 Non-Radioactive Cytotoxicity kit (Promega). Alternatively, synthetic PSMs were incubated for 10 min at room temperature with serum, serum gel filtration fractions or lipoproteins and subsequently tested in the neutrophil lysis assay.

Gel filtration of PSM-FITC with serum

To study the PSM interaction with serum components, synthetic PSMα3 was labeled with fluorescein isothiocyanate (FITC), by incubating 1 mg/ml PSMα3 with 100 µg/ml FITC in 0.1 M sodium carbonate buffer (pH 9.6) for 1 hour at 37°C. FITC-labeled PSMα3 (PSMα3-FITC) was separated from unbound FITC using a HiTrap desalting column (Amersham Biosciences). To determine the retention volume of PSMα3 or PSMα3-FITC alone, 100 µg/ml was loaded onto a Superdex 200 10/300GL (GE Healthcare) equilibrated with PBS. The interaction with serum components was studied by incubating 100 µg/ml PSMα3-FITC with 10% human serum, 2 mg/ml HDL or 1 mg/ml LDL. In some experiments, 500 µl fractions were collected after column passage and fluorescence was quantified with a platereader fluorometer (Flexstation, Molecular Devices). Protein content was measured at OD280 nm and the FITC-extinction was measured at OD_{492nm} on an AKTA explorer (Amersham). Serum and lipoproteins had minimal auto extinction at OD_{492nm} allowing measurement of the association of PSMα3-FITC with serum components at $OD_{492nm.}$ The column was calibrated using the HMW Calibration kit (GE Healthcare) containing Thyroglobulin (669 kDa), Ferritin (440 kDa), Aldolase (158 kDa), Conalbumin (75 kDa) and Ovalbumin (43 kDa). In some experiments, 100% heat inactivated serum was separated by gel filtration and subsequently 0.5 ml fractions were collected for further analysis. In other experiments, 100 µg/ml PSMα3-FITC in PBS containing 0.1% sodium-desoxycholate (PBS-DOC) was applied to the gel filtration column equilibrated with PBS-DOC.

Serum pull-down assays and proteomics

PSMα1 and PSMα3 were immobilized on CNBr-Sepharose (Amersham) fast flow according to the manufacturer's suggestions. The coupling density was 2.5 mg/ml of resin for both PSMs. Following quenching of excess reactive groups with 1 M ethanolamine (pH 8.0) for

2 h at room temperature, the affinity resins were washed and stored as 50% slurries in PBS. To test for binding to serum proteins, heat inactivated human serum was diluted 1:20 in PBS and mixed individually with 20 µl of each affinity resin in a total volume of 0.5 ml. After a 30 min incubation at room temperature under vigorous agitation, the resins were pelleted by centrifugation at 3500 g for 2 min, and washed five times with 1 ml of PBS or PBS containing 0.1% Tween20 (PBST). After the last wash, each resin was resuspended in 20 µl of 2× Laemmli sample buffer, mixed briefly, and heated at 95°C for 5 min. Following sample preparation, the proteins contained in each sample were separated by 12.5% SDS-PAGE and visualized by instant blue staining. For protein identification, the bands of interest were excised from the gel and subjected to in-gel proteolysis by trypsin as described by [38]. The resulting tryptic fragments were extracted, separated by capillary liquid chromatography (LC), and characterized by tandem mass spectrometry (MS/MS). Proteins were identified by comparing the observed fragmentation ion patterns against a data base of human proteins using the MASCOT software package.

HPLC analysis

Analytical HPLC was performed using an automatic HPLC system (Shimadzu) with an analytical reversed-phase column, an UV detector operating at 214 nm with a flow rate of 0.75 mL/min. A Phenomenex Gemini C18 (110 Å, 5 µm, 250×4.6 mm) column was used. TFA buffers (buffer A: H_2O:MeOH, 95:5, v:v; buffer B: MeOH:H_2O, 95:5, v:v, both containing 0.1% TFA). Elution was effected with either a linear gradient from 100% A to 100% B over 60 min or a linear gradient from 40%A to 100%B over 45 min. The molecular mass in the respective peak was determined using electrospray mass spectrometry (ESI-MS), which was performed on a Thermo Finnigan LCQ DECA XP MAX ion trap mass spectrometer and respective PSMs were identified and matched in both retention time and mass synthetic PSMs. δ-toxin and PSMα2 were not separated in both systems.

Generation of *psm*α promoter-GFP construct

The *psm*α promoter-GFP construct was made similar to the method described previously [39]. Shortly, 270 bp upstream of the *psm*α1 start codon (excluding the SD sequence) was amplified by PCR using Phusion polymerase (Finnzymes), using the following primers 5'-AGAATTCGCATGCCTAACGTGTTATTCGTTTTAAACTTAT-3') and 5'-GGATCCTCTAGATTTGCTTATGAGTTAACTTCATTGTA-3' (Life Technologies) and chromosomal DNA from strain Newman as template. Purified PCR products were digested with XbaI and EcoRI (New England Biolabs) and ligated in the likewise digested shuttle vector pSK236-GFP-uvr [39]. The ligation mixture was introduced in *E. coli* Top10F' using the CaCl2 method [40]. Colonies were checked for GFP expression using an ImageQuant LAS4000 (GE Healthcare Life Sciences), and positive clones were checked by restriction analysis and sequencing of the insert. Correct plasmids were introduced into *S. aureus* strain RN4220 by electroporation as described by [41], re-isolated, and introduced by electroporation in *S. aureus* MW2 and the MW2 *agr* knockout.

GFP reporter assays

To measure the GFP expression of the *S. aureus* strains MW2 the MW2 *agr* KO in culture, the strains containing the ***psm***α promoter-GFP construct were grown in MHB with 10 µg/ml chloramphenicol. Overnight cultures were diluted 1:10000 and grown to an OD_{660nm} of

0.1. The cultures were transferred to a clear 96 well flat bottom polystyrene tissue culture plates (Greiner) using 150 µl culture/well. The plate was grown in a Fluostar Omega plate reader (BMG labtech) at 37°C with constant double orbital shaking (400 rpm) in between measurements. Both the absorbance at 660 nm and GFP fluorescence (excitation 485 nm/ emission 520 nm) were measured every 10 minutes for each well. The signal from 4 identical wells was averaged and corrected for blank wells containing only medium.
To detect GFP expression in neutrophils after phagocytosis of S. aureus, an overnight culture of *S. aureus* MW2, containing the *psm*α promoter-GFP construct, was diluted 10000× in MHB and allowed to grow till OD_{660nm} 0.08. Then, the bacteria were collected by centrifugation and washed once in PBS. Bacteria were opsonized in 10% human serum in RPMI for 5' at 37°C, washed once in PBS and resuspended in RPMI/HSA to an OD_{660nm} of 0.01, which corresponds to 5×10^6 bacteria/ml. A three-channel flow cell for inverted microscopes (24×50 mm borosilicate cover glass, size 1,5 (VWR International BV, The Netherlands) mounted at the underside) with channel dimensions of 1×4×40 mm was assembled and sterilized as described [42]. To promote adherence of neutrophils, the channels were coated with 25% human serum in RPMI by flowing in the serum, stopping the flow, clamping off the channels on both sides, and incubating overnight at 4°C. Before introduction of neutrophils, the channels were flushed with RPMI to remove unbound serum. Freshly isolated neutrophils were diluted to 5×10^5/ml and 200 µl was injected into each channel of the serum-coated flow cell. Neutrophils were allowed to adhere for 30 minutes at RT, after which the opsonized bacteria were injected in the channel at a ratio of 10 bacteria: 1 neutrophil. The channel was clamped on both sides, the tubes were cut and the flow cell was transferred to the microscope stage. Neutrophils and bacteria were imaged using a Leica TSC SP5 inverted microscope equipped with a HCX PL APO 40×/0.85 objective (Leica Microsystems, The Netherlands). The microscope was encased in a dark environment chamber that was stably kept at 37°C. 15 minutes after phagocytosis images were acquired using the camera every 5 minutes in both the bright field and the GFP channel (I3 filter cube) for 3 hours to follow GFP production. In post processing the bright field image was not altered and the green channel was adjusted in LAS AF (Leica) to contrast +10, brightness −10 and gamma 1.3 to reduce the green background before merging of both channels.

Results

Activation and lysis of neutrophils by staphylococcal culture supernatants is inhibited by human serum

Inflammatory PSM activities are generally studied in normal culture medium without the addition of serum [4], [7], [10], [16]. We hypothesized that constituents of human serum could inhibit *S. aureus* virulence by interfering with PSM activity at sites of infection. First we studied the influence of serum on the FPR2-stimulatory capacity of PSMs produced by S. aureus. Therefore, we used an FPR2-transfected HL-60 cell line (HL-60/FPR2), and examined the activating capacity of culture supernatants of wild type (WT) MW2 strain and an isogenic *agr* knockout (MW2 *agr* KO) strain, which is described not to produce PSMs [10] (**Figure 1A**). Without serum, the supernatant of WT MW2 activated HL-60/FPR2 cells very potently, in contrast to the supernatant of the MW2 *agr* KO strain. Addition of 1% normal human serum reduced the activity of the supernatant of WT MW2 to the level induced by the MW2 *agr*

KO supernatant. The FPR2-activation by the MW2 *agr* KO supernatant was not affected by the addition of human serum, suggesting that human serum specifically inhibits the PSM-induced FPR2-activation. The inhibition of PSM-induced FPR2 activation by human serum was not species specific, as the addition of mouse, rabbit or bovine serum also inhibited the activation of HL-60/FPR2 cells by the WT MW2 supernatant (data not shown).

Next to the FPR2-activating capacity of *S. aureus* culture supernatants, the MW2 supernatant

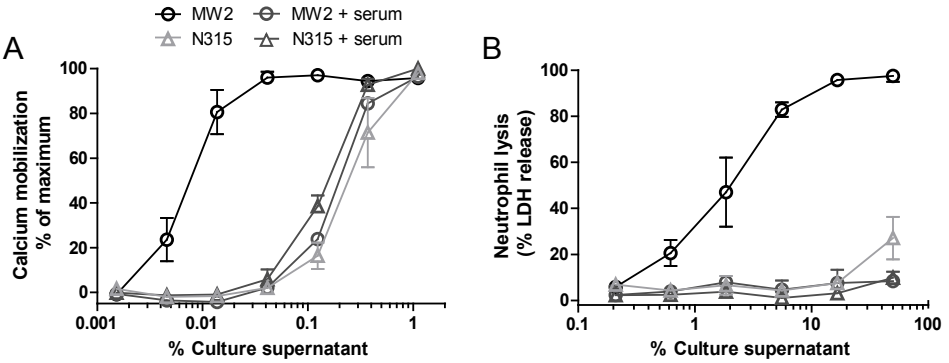

Figure 1. Human serum inhibits the activity of PSMs in culture supernatants.
(A) Dose-dependent calcium mobilization of HL-60/FPR2 cells by culture supernatants of *S. aureus* strains MW2 and MW2 *agr* KO, with or without preincubation with 1% heat-inactivated human serum. (B) Dose-dependent neutrophil lysis by *S. aureus* culture supernatants with or without preincubation with 5% heat-inactivated human serum. Neutrophil lysis was measured through LDH release. Data represent means ± SEM of three independent experiments.

has also been described to very potently lyse isolated human neutrophils [7]. Therefore, we investigated the effect of serum on the culture supernatants of the same *S. aureus* strains in their capacity to lyse neutrophils. Indeed, the culture supernatant of WT MW2 very potently lysed human neutrophils, as measured by the release of LDH, in contrast to the supernatant of the MW2 *agr* KO (**Figure 1B**). Also in this assay, 5% human serum completely abrogated the lysis of human neutrophils induced by the culture supernatant of WT MW2, while leaving the effect of the MW2 *agr* KO supernatant uninfluenced. *Agr* not only regulates the production of PSMs, but also controls the expression of other toxins, for instance the alpha toxin gene (*hla*). Based on the known functions of PSMs, especially their FPR2 activating capacity, we hypothesize that PSMs are the main effectors causing the differences in cell activation and lysis between the WT MW2 and the MW2 *agr* KO supernatants. Therefore, we think that PSMs are specifically inhibited by human serum. As described by others [7], [10], we also observed that the percentage *S. aureus* MW2 supernatant needed for neutrophil lysis is 1000 times higher than that needed for FPR2 activation.

Not only *S. aureus* strain MW2, but also strains USA300 and Newman are known to produce high levels of PSMs. In contrast, strains N315 and COL have been described to produce low levels of PSMs [7], [10]. When we examined the activity of the supernats of these *S. aureus* strains, our results correlated with the described production of PSMs. FPR2-induced cell activation and neutrophil lysis were only induced by the supernatants of the high PSM-producing *S. aureus* strains. Importantly, these activities were also potently inhibited by human serum (**Figure S1A** and **S1B**). Our findings thus suggest that human serum inhibits

the FPR2-activation and neutrophil lysis induced by *S. aureus* produced PSMs.

Human serum inhibits the PSM-induced activation of neutrophils

To demonstrate that human serum indeed targets PSMs present in the supernatant of S. aureus, we also tested its activity on synthetic PSMs. All *S. aureus* core genome-encoded PSMs were tested, with the exception of PSM*mec*, for FPR2 stimulatory activity in the presence and absence of human serum. Pre-incubation of pure synthetic PSMs with 0.1% human serum significantly inhibited the ability of all PSMs to elicit calcium mobilization in neutrophils, whereas control stimuli, fMLP, IL-8 and C5a, were not inhibited (**Figure 2A**). The supernatant of *S. aureus* strain MRSA252 containing PSM*mec* was also inhibited by human serum. Although not examined for synthetic PSM*mec*, there is no reason to believe that human serum acts differently to PSM*mec* than to other PSMs. To investigate the kinetics of serum-induced PSM-inactivation, the incubation time of PSMs with serum was varied from 0 to 1800 sec, before testing in a calcium mobilization assay using HL-60/FPR2 cells. 100 nM PSMα3 was inactivated by 0.1% serum within seconds, whereas inactivation of a 5 times

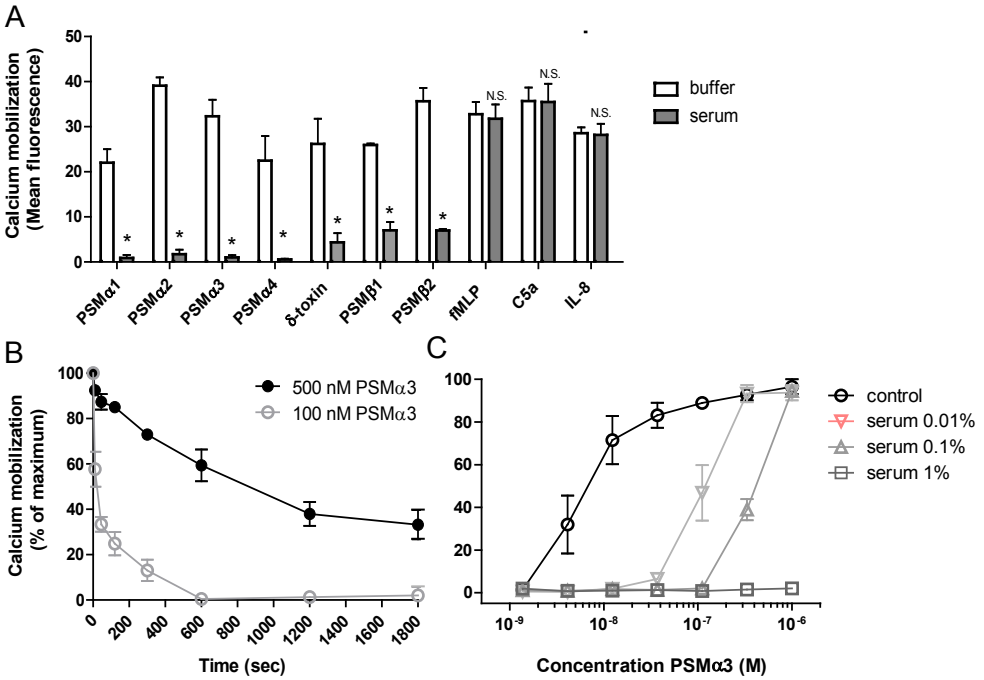

Figure 2. Human serum inhibits PSM-mediated neutrophil activation.
(A) Calcium mobilization of human neutrophils. Neutrophils were stimulated with 10^{-6} M PSMα1, 10^{-7} M PSMα2, 10^{-7} M PSMα3, 10^{-6} M PSMα4, 3×10^{-6} M δ-toxin, 10^{-5} M PSMβ1, 10^{-5} M PSMβ2, 10^{-9} M fMLP, 10^{-10} M C5a and 10^{-10} M IL-8, all preincubated with or without 0.1% heat-inactivated human serum, before calcium mobilization was measured by flow cytometry. *, p<0.001; N.S., not significant. (B) Time-dependent inhibition of PSMα3-mediated calcium mobilization of HL60/FPR2 cells. PSMα3, 100 nM or 500 nM, was preincubated with 0.1% human serum and calcium mobilization was measured at different time-points by flow cytometry. (C) Dose-response curves for calcium mobilization in HL-60/FPR2 cells induced by PSMα3 or serum-treated PSMα3. Data represent means ± SEM of at least three independent experiments.

higher PSM concentration was clearly delayed (**Figure 2B**). Higher concentrations of PSMs of up to 1 µM could be fully inhibited by 15 minutes pre-incubation with 1% serum (**Figure 2C**). These results indicate a time- and dose-dependent inhibition of PSM-induced FPR2-activation by human serum.

Human serum inhibits the *S. aureus* PSM-induced neutrophil lysis

In order to investigate the ability of human serum to inhibit PSM-induced neutrophil lysis, we screened the synthetic PSM peptides preincubated with serum for neutrophil lysis. For this, we used a concentration range of 400 nM to 100 µM for each PSM. These concentrations are biologically relevant, as Wang *et al* [7] have described that the CA-MRSA MW2 and USA300 strains produce δ-toxin up to 30 µM in an overnight culture. Other α-type PSMs are typically produced at somewhat lower concentrations, ranging from 5 µM to 15 µM. The cytolytic activity of PSMα1, PSMα2, PSMα3, and δ-toxin towards neutrophils was completely abrogated in the presence of serum (**Figure 3**). In addition, the lysis of peripheral blood mononuclear cells by synthetic PSMα3 was inhibited by human serum (**Figure S2A**). To investigate whether other staphylococcal toxins may also be inhibited by human serum, we tested the effect of serum on PVL-mediated lysis. The lysis of neutrophils induced by PVL toxin was not affected by human serum (**Figure S2B**), implicating that serum does not generally inhibit all toxin-mediated cell lysis. As shown previously [7], α-type PSMs are very potent in cytolysis; however, in our hands neutrophil lysis by β-type PSMs under

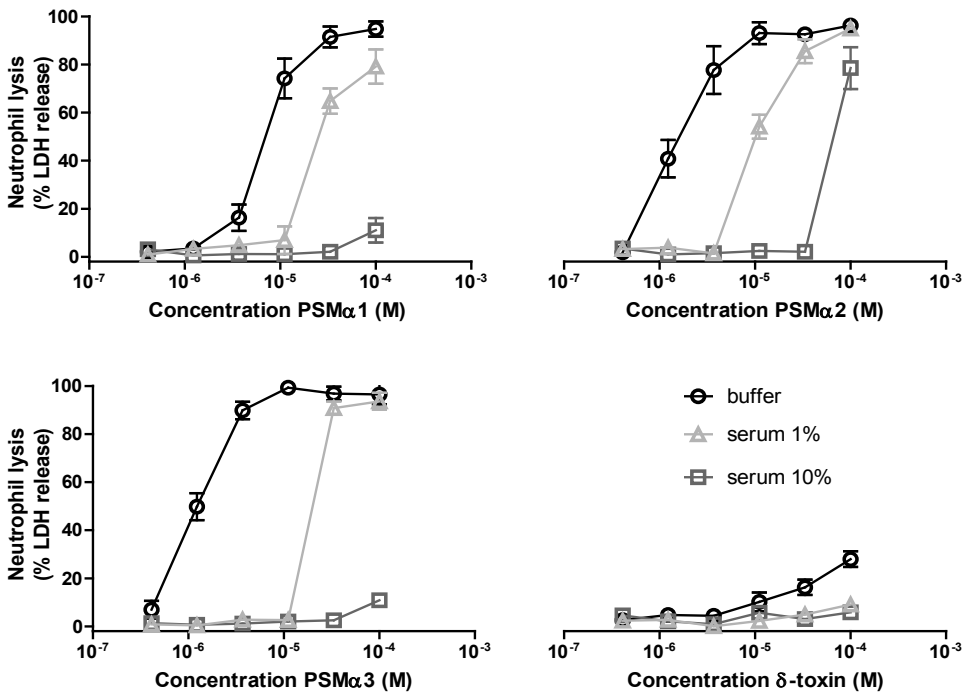

Figure 3. Inhibition of PSM-mediated neutrophil lysis. Dose-dependent neutrophil lysis by synthetic PSMα1, PSMα2, PSMα3, and δ-toxin (400 nM to 100 µM), preincubated with or without 1% or 10% human serum. Neutrophil lysis was measured through LDH release. Data represent means ± SEM of three independent experiments.

physiologically-relevant concentrations was not observed. Collectively, we demonstrated that serum inhibits the FPR2-activating and neutrophil lysing capacity of all *S. aureus* PSMs.

PSMs interact with serum lipoproteins

To capture the serum components able to inactivate PSMs, we coated CNBr-Sepharose beads with synthetic *S. aureus* PSMα1 or PSMα3 and incubated the generated beads with 20% heat-inactivated serum. Following extensive washing, the specifically-bound proteins were eluted, analyzed by non-reducing SDS-PAGE and visualized by Instant Blue staining (**Figure 4A**). Both PSMα1 and PSMα3 bound to a protein of approximately 25 kDa, which was identified by mass spectrometry as ApoA1. Interestingly, when we performed the same experiment and additionally washed the PSM-coated beads after serum incubation with a detergent, ApoA1 was not longer detected. We were unable to detect a direct interaction of immobilized PSMs with recombinant ApoA1 using ELISA or Surface Plasmon Resonance (data not shown). ApoA1 is however the major protein constituent of high density lipoprotein (HDL), which could possibly bind PSMs. Serum lipoproteins are complex particles with a neutral core containing triglycerides and cholesterol and covered by an amphipathic monolayer of phospholipids and unesterified cholesterol. The Apo-protein components bind to the surface of the particles and are either restricted to particular lipoproteins or freely exchangeable across lipoprotein categories. Therefore, the inability of PSMs to bind to ApoA1 in the presence of a detergent most likely indicates that PSMs bind to the lipid content of the HDL particle rather than a specific interaction with ApoA1. To test this hypothesis, we performed serum size exclusion assays with FITC-labeled PSMα3 (PSMα3-FITC) to find the PSM-binding components in serum. The FITC-labeling did not affect the function of the PSMα3 molecule, as PSMα3-FITC was as potent as unlabeled PSMα3 in the FPR2-activation of neutrophils (**Figure S3**). At first, the retention volume of PSMα3-FITC

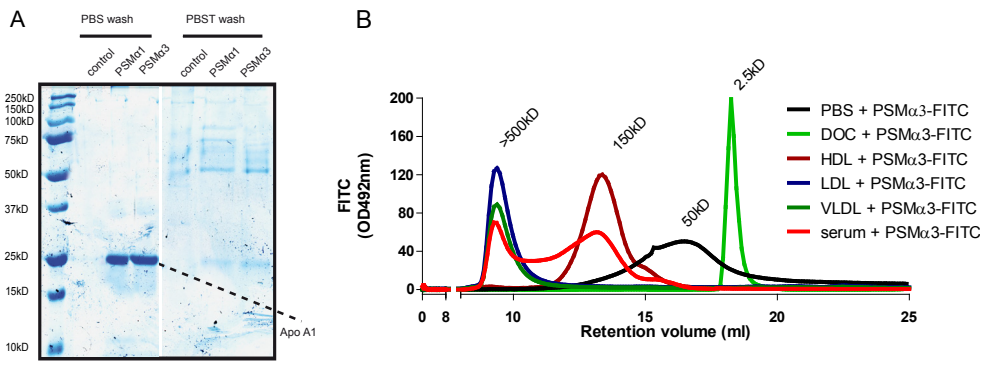

Figure 4. PSMs associate with serum lipoproteins. (A) Serum pull down assay with PSMα1 and PSMα3 coupled to CNBr beads. Beads were extensively washed with PBS or PBS with tween (PBST). Serum proteins bound and eluted from the beads were visualized by SDS-page followed by instant blue staining. Protein bands specifically appearing in the PSMα1 and PSMα3 lane were identified by MALDI-TOF mass spectrometry as ApoA1. (B) Gel filtration association assay. Comparison of absorption (OD492 nm) profiles of 100 µg/ml PSMα3-FITC pre-incubated with PBS, 10% human serum, 1 mg/ml HDL, 1 mg/ml LDL or 1 mg/mL VLDL for 30 min, before separation on a gel filtration column. For monomerization of PSMα3-FITC, the gel filtration column was equilibrated with PBS containing 0.1% sodium deoxycholate (DOC). Representative figures of two independent experiments.

was determined; PSMα3-FITC behaved as an aggregate of peptides in a physiological buffer, resulting in a broad peak of approximately 50 kDa (**Figure 4B**). In contrast, when a similar size exclusion run was performed in the presence of a detergent, a peak of monomeric PSMα3-FITC of approximately 2,5 kDa was observed. This is in line with the first-described discovery of PSMs, where similar poly-peptide complexes were observed when PSMs were extracted from *S. epidermidis* culture supernatant [17]. Multimeric aggregations have also been reported for *S. aureus* δ-toxin with molecular weights of 5–200 kDa, with the lower molecular weights observed at extreme pH or in organic solvents, and the larger molecular weights observed in water [18], [19]. Interestingly, when PSMα3-FITC was incubated with human serum prior to the size exclusion assay, the 50 kDa peak shifted towards two peaks, one of 150 kDa and one larger than 500 kDa, suggesting an association with HDL, known to run at 150 kDa, and low density (LDL) or very low density (VLDL) lipoproteins, known to run at higher than 500 kDa. To demonstrate that indeed PSMα3-FITC associated with lipoproteins in serum, we purified HDL and LDL from serum and repeated the size exclusion experiment using PSMα3-FITC preincubated with purified HDL or LDL. When PSMα3-FITC was preincubated with HDL, detection of the FITC signal shifted from a molecular weight of 50 kDa towards 150 kDa, whereas the signal of PSMα3-FITC preincubated with LDL, which, as VLDL, represents high molecular weight particles, shifted to the void volume of the gel filtration column. Importantly, the fluorescent 150 and >500 kDa peaks of the size exclusion chromatograms of PSMα3-FITC and serum correspond and are overlapping with the peaks of the size exclusion chromatograms of PSMα3-FITC with HDL and PSMα3-FITC with LDL or VLDL. As the interactions of PSMα3-FITC with HDL, LDL or VLDL do not shift the retention times of these lipoproteins on the gel filtration column, it appears that only monomeric PSMα3-FITC molecules interact with the lipoprotein particles (**Figure S4**). In conclusion, several serum lipoproteins can associate with PSMs.

Lipoproteins antagonize PSMs

We further investigated whether serum lipoproteins, by binding PSMs, are responsible for antagonizing the functions of PSMs. Therefore, we depleted normal human serum for lipoproteins by density ultracentrifugation. Compared to untreated serum, lipoprotein-depleted serum lost the ability to inhibit the PSMs induced lysis of neutrophils (**Figure 5A**), indicating that lipoproteins are indeed the serum components that inhibit PSMs. Next, we tested the effect of purified HDL and LDL on PSM-function. Normal HDL or LDL protein levels in serum are between 1 and 1.3 mg/ml. Purified HDL and LDL at physiologically relevant concentrations, representing serum levels of 1 and 10%, inhibited the PSM-mediated lysis of neutrophils (**Figure 5B**). In addition, HDL and LDL inhibited the calcium mobilization induced by synthetic PSMs (**Figure 5C**). Addition of recombinant ApoA1 or ApoB1 did not inhibit the PSM-induced activation (**Figure S5A**) or lysis (**Figure S5B**) of neutrophils. To determine the serum lipoprotein with the highest neutralizing capacity, we separated human serum by size exclusion chromatography. Subsequently, the separate fractions were tested for inhibition of a high or a low lethal dose of PSMα3 (**Figure 6**). At the high lethal dose (50 μM) only the fractions containing HDL inhibited neutrophil lysis, whereas at a lower still lethal dose (10 μM) also LDL/VLDL could inhibit the lysis of neutrophils, indicating that HDL is the most potent inhibitor within human serum. Next, human serum was spiked with PSMα2, and HDL, LDL, VLDL and lipid-free serum were isolated by density ultracentrifugation and

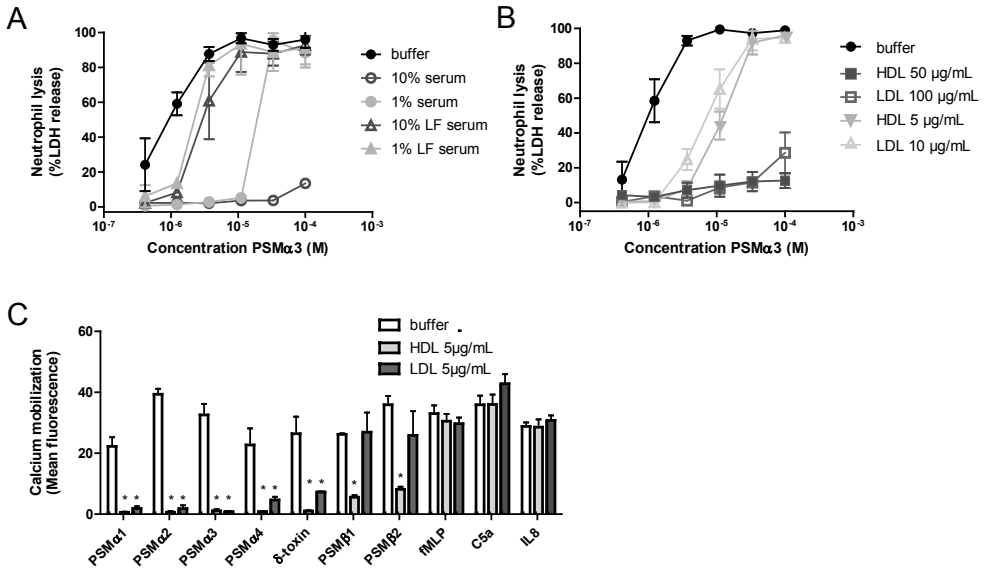

Figure 5. Serum lipoproteins inhibit PSM-mediated neutrophil lysis and activation. (A) Dose-dependent neutrophil lysis by synthetic PSMα3 preincubated with 1% and 10% human serum or human lipid-free (LF) serum or preincubated with (B) 5 and 50 µg/ml HDL or 10 and 100 µg/ml LDL (concentrations are based on protein content). PBS was used as buffer control. Neutrophil lysis was measured via LDH release. (C) Calcium mobilization of human neutrophils. Neutrophils were stimulated with with 10^{-6} M PSMα1, 10^{-7} M PSMα2, 10^{-7} M PSMα3, 10^{-6} M PSMα4, 3×10^{-6} M δ-toxin, 10^{-5} M PSMβ1, 10^{-5} M PSMβ2, 10^{-9} M fMLP, 10^{-10} M C5a and 10^{-10} M IL-8, all preincubated with or without 5 µg/ml HDL or LDL, before calcium mobilization was measured by flow cytometry. *, p<0.001; N.S., not significant.

analyzed by HPLC (**Figure 6C**). Approximately 80% of the spiked PSMα2 was found in the HDL-containing fractions, whereas approximately 15% and 5% of PSM was recovered in LDL- and VLDL-containing fractions, respectively. We did not detect PSMs in the lipid-free serum. Finally, we investigated whether PSMs could also be recovered in serum constituents when *S. aureus* was cultured in blood. Therefore, we cultured *S. aureus* MW2 overnight in freshly drawn lepirudin-anticoagulated blood, isolated the HDL fraction from the clarified blood the following day and analyzed it by HPLC/LC MS. **Figure 6D** shows the presence of PSMα1, PSMα2, PSMα3, PSMα4 and δ-toxin in the purified HDL fraction, indicating that PSMs produced by viable *S. aureus* could be neutralized by lipoproteins in a blood environment

PSMα is produced by *S. aureus* upon phagocytosis by neutrophils

Thus far, our data strongly suggest that the functional properties of PSMs produced by *S. aureus* in serum as well as in whole blood are inhibited by lipoproteins. These results conflict with literature describing PSMs as key virulence determinants for CA-MRSA, with regard to their cell lytic properties [7]. Therefore, we hypothesized that PSMs rather act as toxins in an environment devoid of lipoproteins, such as the neutrophil phagosome. To test whether PSMs could act intracellular and are produced by *S. aureus* after uptake by human neutrophils, we generated a construct in which the promoter of the *psm*α operon drives GFP expression. This *psm*α promoter-GFP construct was transformed into *S. aureus*

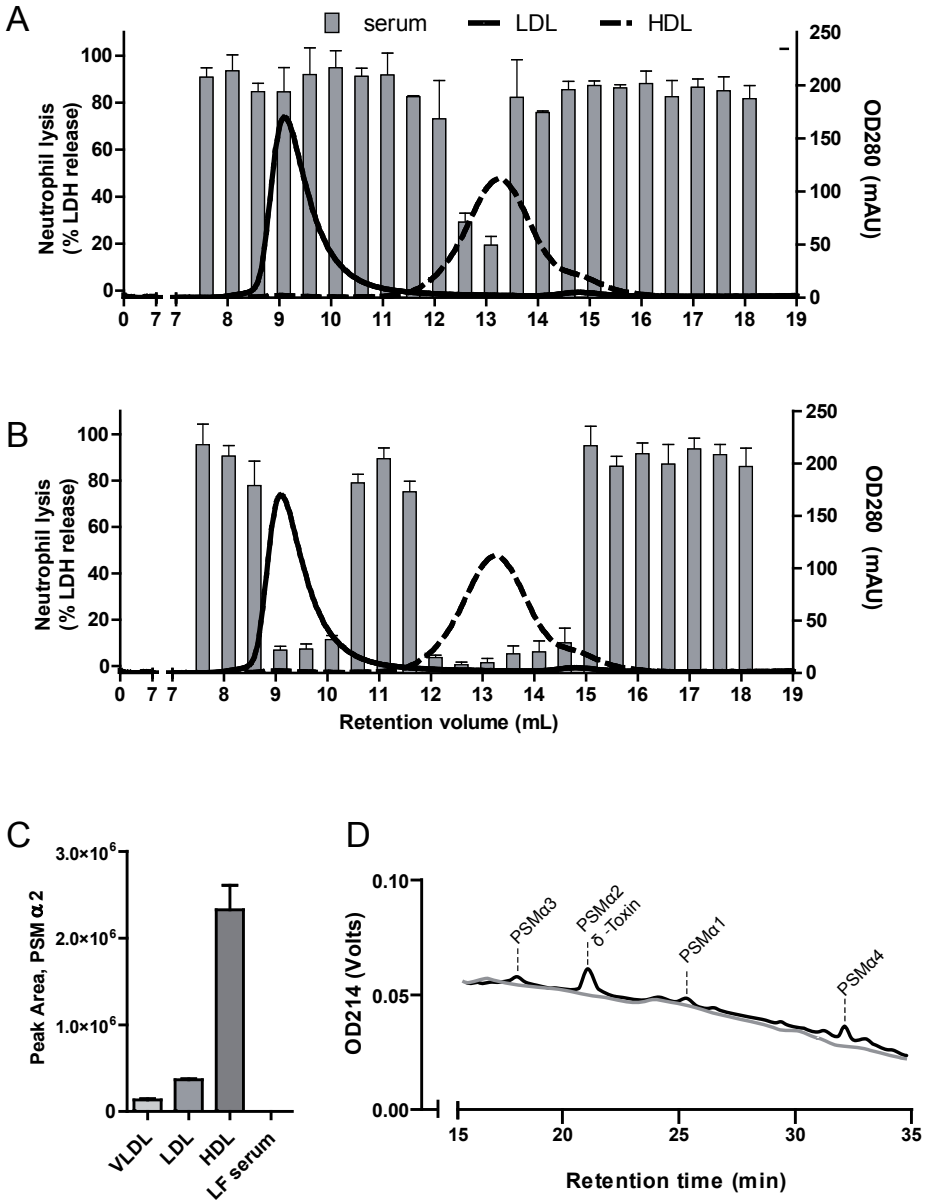

Figure 6. Identifying the most potent inhibitor of PSMs in serum.
Functional screening of serum fractions, isolated by gel filtration, for the inhibition of neutrophil lysis. Serum fractions were incubated with (A) 50 µM or (B) 10 µM of PSMα3 before addition to neutrophils and lysis was measured via LDH release. Data represent means ± SEM of three independent experiments. (C) PSM concentration measured in isolated lipoprotein fractions after spiking human serum with 0.5 mg/ml PSMα2. PSM concentration was measured by reverse phase-HPLC and represents the mean of the area under the curve of PSMα2 of 3 independent experiments. (D) Measurement of the concentration PSM by HPLC in isolated HDL fraction from an overnight whole blood culture of the *S. aureus* MW2 strain (black line) or control (no bacteria; gray line). HDL fractions were subjected to HPLC and absorbance at 214 nm was obtained. Respective PSM were identified by LC/MS, δ-toxin and PSMα2 were contained in the same peak in this assay condition.

MW2, the bacterium was cultured over time, and the activation of the *psm*α promoter was monitored with a fluorescent plate reader. As expected for an *agr*-controlled expression, the GFP expression was detected during the late logarithmic growth phase. Under these conditions, we did not observe expression of GFP when we introduced the construct in the MW2 *agr* KO strain (data not shown). Next, we examined GFP expression upon phagocytosis of MW2 bacteria containing the *psm*α promoter-GFP construct by neutrophils. Therefore, the bacteria were first cultured to very early logarithmic phase to prevent initial activation of the *psm*α promoter. Then, they were opsonized with human serum, presented to neutrophils adherent to a flow cell, and bacterial fluorescence upon phagocytosis by neutrophils was assayed over time with a fluorescent microscope. Fluorescence within neutrophils was observed 45 min to 2 hours after phagocytosis (**Figure 7+Video S1)**, indicating activation of the *psm*α promoter and thus potential production of *psm*α inside neutrophils. We did not observe fluorescence for bacteria found outside neutrophils, except when they formed dense micro-colonies (data not shown). These data indicate potential *psm*α expression inside neutrophils after phagocytosis of *S. aureus.*

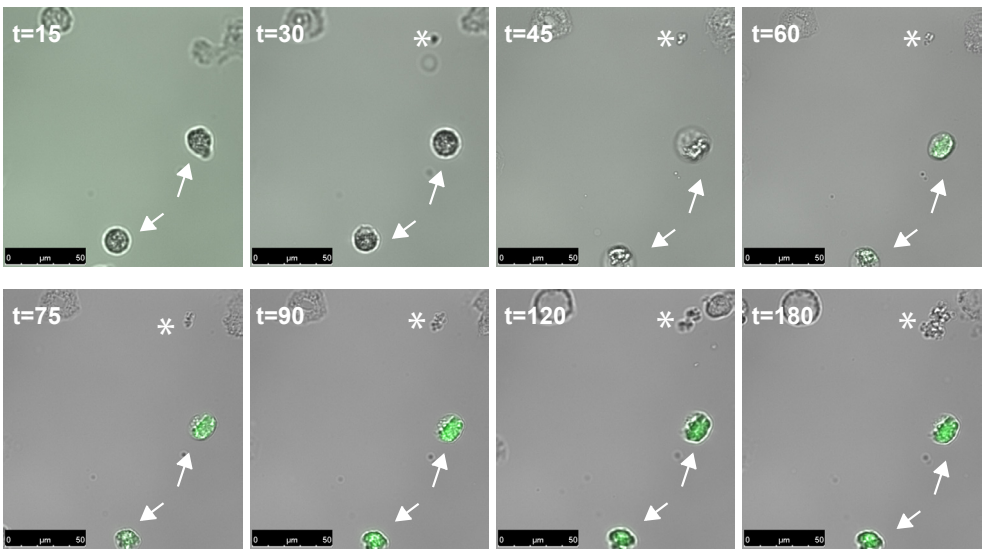

Figure 7. Rapid upregulation of PSMα expression after phagocytosis. *S. aureus* MW2 containing the PSMα promoter-GFP construct was incubated with neutrophils, and fluorescence as a measure for *psm*α expression was monitored over time (0–3 hours). Arrows indicate neutrophils which have phagocytozed S. aureus. (*) Indicate growing reporter bacteria outside of the cells. Bars: 50 µm.

Discussion

Serum transports the humoral components of the innate immune system throughout the human body. Apart from the complement system and the coagulation system, the lipid transport system has been found to play a role in innate immunity as well. ApoB1, the major protein constituent of VLDL and LDL, can sequester AIP and thereby prevent quorum sensing of *S. aureus* [15]. We show in this study that serum lipoproteins dampen inflammatory

over-stimulation and inhibit the cytolytic activities of the *agr*-controlled PSMs. PSMs are sequestered by serum lipoprotein particles and thereby lose their functional properties, which may allow their clearance from the system, analogous to LPS sequestration by HDL [20]. This has great implications for our understanding of PSM function during infection. All classes of plasma lipoproteins are normally present in interstitial tissue fluids at approximately 6% of the plasma concentration for VLDL to 20% for HDL [21]. This interstitial lipoprotein concentration even increases in case of inflammation. Although PSMs can lyse neutrophils and *S. aureus* can produce PSMs in very large quantities [6], [7], the majority of these PSMs will most likely rapidly be neutralized by serum lipoproteins, making it unlikely that the high concentrations required to cause neutrophil lysis can be reached. Therefore, we propose that the role of PSMs as important secreted extracellular toxins should be reconsidered.

We do not rule out a possible role for the membrane-lytic effects of PSMs within neutrophils after phagocytosis of S. aureus. Intracellular lysis could account for the fact that experimental mouse studies with PSMs knockout *S. aureus* strains show reduced virulence [4], [7]. It has recently been described that the concentration of AIP, responsible for gene transcription upon quorum sensing, can reach the critical concentration within cells, allowing the *agr* system to function within the neutrophil [22], [23]. In line with these studies, we show that the promoter for the *psm*α operon is activated after phagocytosis of *S. aureus* by neutrophils. This strongly suggests that PSMα cytolytic peptides are produced within the neutrophil phagosome and may have a function there. Since the neutrophil phagosome is devoid of lipoprotein particles, PSMs may act intraphagosomally and allow *S. aureus* to escape from the phagosome and thereby avoiding killing. Similarly, staphylococcal alpha toxin (Hla), has been demonstrated to allow endosomal escape after phagocytosis [24], [25]. However, further research is needed to test whether PSMs have a similar mechanism of action. Next to the neutrophil phagosome, other niches within the human body lacking serum lipoproteins may allow for extracellular PSM functions.

PSMs are thought to lyse cells by disrupting the cell membrane. *S. aureus* delta toxin is proposed to form a cation-selective membrane pore with a central hydrophilic channel by the multimerization of 6 monomers and an outer hydrophobic interaction with membrane lipid [18], [26]. The intrinsic structural properties of the amphipathic alpha helix of PSMs likely mediate a similar multimerization in response to a lipid bilayer. This implies that PSMs randomly insert into lipid membranes without specific targeting of host cells. In line with this, PSMγ and PSMδ from S. epidermidis have been shown to perforate synthetic POPC/POPG ((1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine)/(1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)]) lipid vesicles [27]. PSMs thus seem to target lipid layers without the presence of membrane proteins, suggesting that there is no species specificity. This is in contrast to other staphylococcal cytolytic toxins, such as PVL, which show strong human specificity [28]. Staphylococcal two-component toxins are proposed to induce receptor-mediated lysis, which explains why they are not inhibited by human serum lipoproteins. PSMs seem to have high affinity for lipids. Therefore, it is not surprising that their biological actions are inhibited by the major humoral lipid transportation system, the lipoprotein particles. The two most abundant serum lipoprotein particles are HDL and LDL. Although the normal serum concentration of LDL (ApoB1 1.6 mg/ml) is higher than the HDL (ApoA1 1.2 mg/ml), the combined surface of the HDL particles exceeds 3 times the surface

of LDL particles. The surface size difference therefore most likely accounts for the higher inhibitory potential of HDL compared to LDL.

As we clearly show that PSMs not only interact with HDL, but also with LDL and VLDL, we argue that the Apo proteins within the lipoprotein particles do not play a specific role in binding the PSMs. Although we tried, we could indeed not detect a direct interaction between purified Apo-proteins and synthetic PSMs. In addition, PSMs are unable to bind to lipoprotein particles in the presence of a detergent, and PSMs can interact with synthetic lipid vesicles [27]. Therefore, we expect that PSM bind to the lipid contents of the lipoprotein particles without the need for a specific interaction with Apo proteins within the complexes.

PSMs have the capacity of attracting neutrophils in the nanomolar range, exceeding their lytic capacity over a 1000 fold. There is increasing evidence that *S. aureus* can survive inside neutrophils [29] and that intracellular survival contributes to pathogenesis [30]. Neutrophil attraction to the site of infection may thus possibly be advantageous for the bacterium. On the one hand, it has been demonstrated that neutrophils are necessary to control the infection; to subvert neutrophil attraction through FPR2 *S. aureus* can also secrete two FPR2 antagonists, FLIPr or FLIPr-like [12], [13] and prevent excessive PSM-induced neutrophil migration. The higher PSM production of CA-MRSA strains, as compared to HA-MRSA strains, might however tilt the balance in favour of neutrophil migrating towards the infection side. Then, a higher PSM production inside the neutrophils upon *S. aureus* phagocytosis may rescue CA-MRSA from phagosomal killing, contributing to its enhanced virulence.

Serum lipoproteins have a well-known function in dampening immune responses. As for PSMs, lipopolysaccharide (LPS) and lipoteichoic acid can be inactivated by human serum lipoproteins [31]–[33]. Also comparable to PSMs, LPS forms large aggregates in aqueous conditions. Moreover, both PSMs and LPS are taken up by HDL as monomeric molecules, resulting in their inactivation. The process of LPS- and PSM-inactivation by HDL displays different kinetics, since it takes hours for HDL to take up LPS [34], whereas we show uptake of PSMs by HDL within seconds. For LPS, the serum components LPS-binding protein (LBP) and soluble CD14 (sCD14) catalyze the process of LPS transfer. Both LBP and sCD14 bind to the toxic Lipid A moiety of LPS and facilitate the transfer of monomeric LPS to HDL or to cellular expressed CD14, resulting in inactivation of LPS and activation of the LPS receptor TLR4, respectively. Thus far, there is no evidence that a similar serum transfer system exists for the transfer of PSMs to lipoproteins or the recognition of PSMs by FPR2. Our data show that isolated HDL, without the addition of other serum components, is sufficient for PSM transfer to HDL. Additionally, we show no enhancement of the FPR2 activating capacity of PSMs in the presence of lipoprotein deficient serum, as compared to buffer only. These results strongly suggest that no serum component is needed for the PSM inactivation by lipoproteins or the PSM recognition by FPR2.

High production of PSMs by CA-MRSA strains is proposed as the causative factor for the enhanced virulence of CA-MRSA strains, as compared to HA-MRSA strains [6], [7]. Our current study shows strong interaction and neutralization of PSMs by serum lipoproteins, even when PSMs are produced by growing *S. aureus* in whole blood. These results strongly suggest that the contribution of PSMs to the enhanced virulence of CA-MRSA strains is not due to PSMs acting as extracellular toxins. PSMs can only function intracellular or in other lipoprotein-free niches in the body. Low non-toxic concentrations of PSMs might attract neutrophils to the site of infection enabling uptake of S. aureus. Once inside the

cell, production of PSMs might help *S. aureus* in its escape from the phagosome, aiding in its survival and virulence. The antimicrobial activity of PSMs against other bacteria might also create a niche for staphylococcal colonization outside the human body. Future studies are needed to shed more light on the exact functions of PSMs and their contributions to CA-MRSA virulence.

Acknowledgements

We thank Dr. J. Bestebroer for critically reading the manuscript.

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Supporting Information

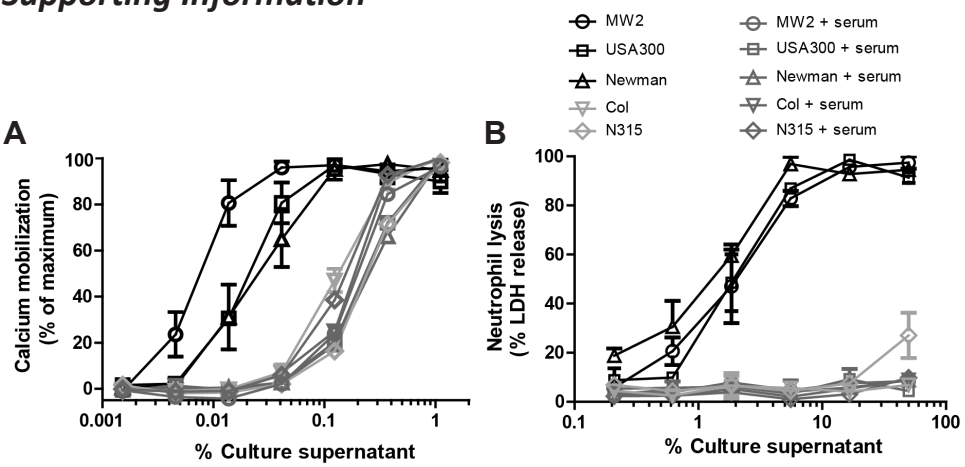

Figure S1. Inhibition of PSM-related functions in culture supernatants by human serum. (A) Dose-dependent calcium mobilization of HL-60/FPR2 cells by the culture supernatants of S. aureus strains USA300 Newman, MW2, COL and N315 with or without preincubation in 1% heat inactivated human serum. (B) Dose-dependent neutrophil lysis by S. aureus culture supernatants with or without 5% human serum. Neutrophil lysis was measured via LDH release. Data represent means ± SEM of three independent experiments.

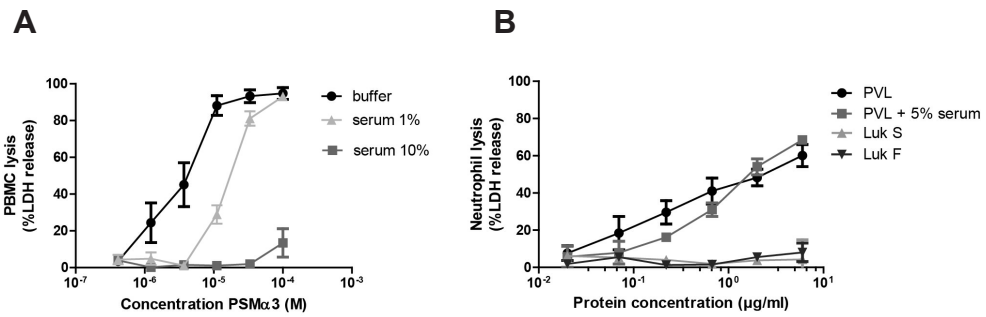

Figure S2. Inhibition of PSM-mediated PBMC lysis and no inhibition of PVL-mediated neutrophil lysis by human serum. (A) Dose-dependent PBMC lysis by synthetic PSMα3 preincubated with or without 1% or 10% human serum. (B) Dose-dependent neutrophil lysis by recombinant PVL protein (LukS and/or LukF) with and without 5% serum. Leukocyte lysis was measured via LDH release. Data represent means ± SEM of three independent experiments.

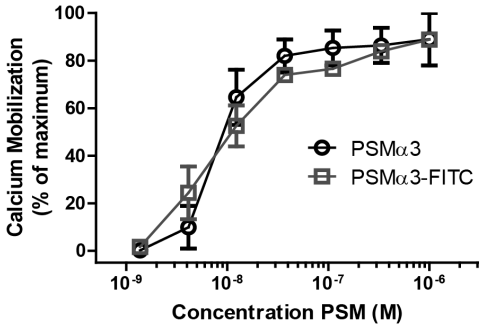

Figure S3. Calcium mobilization of human neutrophils induced by PSMα3 or PSMα3-FITC. Neutrophils were stimulated with a dose response of 1.3×10−9 M to 10−6 M PSMα3 or PSMα3-FITC. Data represent means ± SEM of three independent experiments.

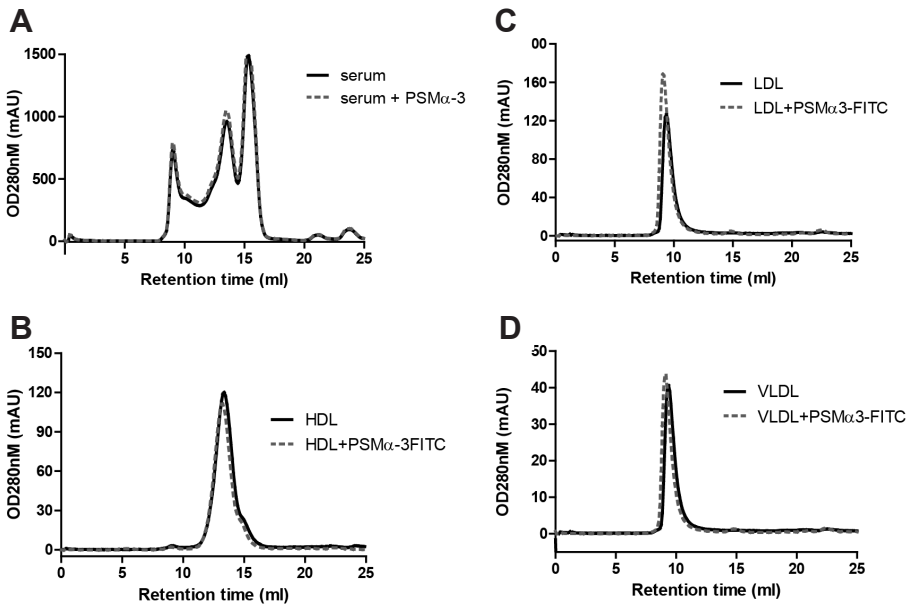

Figure S4. Monomerization of PSM by serum lipoproteins. Gel filtration association assay. Comparison of extinction (OD280 nm) profiles of 100 µg/ml PSMα3-FITC pre-incubated (A) 10% human serum, (B) 1 mg/ml HDL, (C) 1 mg/ml LDL or (D) 1 mg/ml VLDL for 30 min, before separation on a gel filtration column. Representative figures of two independent experiments.

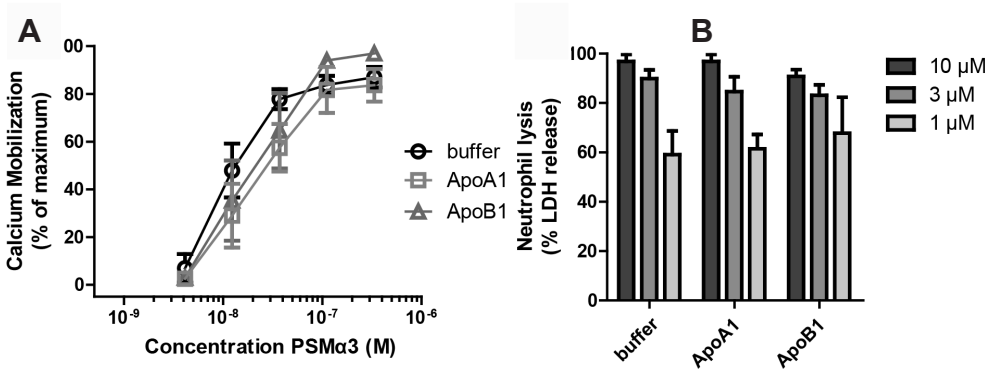

Figure S5. No inhibition of neutrophil activation or lysis by recombinant apolipoproteins. (A) Dose-dependent neutrophil activation by synthetic PSMα3 preincubated with ApoA1 (50 µg/ml) or ApoB1 (50 µg/ml) or buffer, calcium mobilization was measured by flow cytometry. (B) Dose-dependent neutrophil lysis synthetic PSMα3 preincubated with ApoA1 50 µg/ml or ApoB1 50 µg/ml or buffer. Neutrophil lysis was measured via LDH release. Data represent means ± SEM of three independent experiments.

QR code of **supporting movie 1.** ***psmα* expression inside human neutrophils.** Time lapse images showing neutrophils which have phagocytozed *S. aureus* MW2 containing the *psm*α promoter-GFP construct, *psm*α expression (green) was monitored over time. The video starts 15 min after phagocytosis and the images were taken by every 5 minutes. Note, the upper right corner shows a growing *S. aureus* reporter micro-colony which shows no *psm*α expression. No inhibition of neutrophil activation or lysis by recombinant apolipoproteins.

4

HDL Mediate *In Vivo* Protection against Staphylococcal PSMs

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Abstract

Staphylococcus aureus virulence has been associated with the production of phenol soluble modulins (PSMs). PSMs are known to activate, attract and lyse neutrophils. However, in the presence of serum lipoproteins PSMs are functionally inhibited. This study deals with the important question whether neutralization of staphylococcal PSMs occurs *in vivo*, and whether lipoprotein concentrations *in vivo* are sufficient for neutralization of PSMs. In this study, we show enhanced protection of plasma from SR-BI$^{-/-}$ mice against PSM-induced lysis of human neutrophils. High density lipoprotein (HDL) deficiency in LCAT$^{-/-}$ and ABCA1$^{-/-}$ mice resulted in decreased protection against PSM-induced lysis. PSM-induced peritonitis in HDL deficient LCAT$^{-/-}$ mice resulted in an enhanced neutrophil influx, showing that *in vivo* neutralization of PSMs occurs by HDL particles. Finally, plasma from Tangier disease patients characterized by a homozygous ABCA1 mutation and as a consequence containing no plasma HDL, was also less protective against PSM-mediated functions. Therefore, we conclude that lipoproteins present in blood protect against PSMs, the key virulence factor of community-associated methicillin resistant *S. aureus*.

Introduction

Staphylococcus aureus (S. aureus) is a commensal organism in humans and about 30% of healthy individuals are colonized asymptomatically with *S. aureus* in the nostrils [1]. However, *S. aureus* can spread from this location and cause infections ranging from superficial skin infections to fatal necrotizing pneumonia [2]. *S. aureus* is notorious for its ability to acquire resistance to antibiotics, especially methicillin-resistant *S. aureus* (MRSA) is of major concern, as it is among the leading causes of death in the USA [3]. Especially community-associated (CA)-MRSA strains are regarded more virulent than hospital-associated (HA-) MRSA, because they can cause infections in otherwise healthy individuals [4,5]. Among the many virulence factors and immune evasion molecules described for *S. aureus* [6,7], phenol-soluble modulins (PSMs) are one of the few virulence factors contributing to the success of CA-MRSA strains [8,9]. PSMs are small cytolytic core genome-encoded peptide toxins, of which the expression is strictly controlled by the accessory gene regulator (*agr*) system [10]. Although PSMs share a common amphipathic α-helical region, which is thought to enable their cell lytic ability by disrupting the cell membrane, PSMs are categorized in two groups, based on their size [9]. The smaller α-type PSM group, consisting of four PSMα peptides and δ-toxin with a length of 20-30 amino acids, are more cytolytic [9] compared to the larger (44AA) β-type PSMs. Isogenic *psm*α mutants in different genetic backgrounds, have shown significant reduced virulence in bacteremia and skin infection models compared to their wild-type CA-MRSA strains in both mice and rabbits [9,11].

Next to their cytolytic potential, PSMs have the ability to stimulate neutrophils via formylated peptide receptor 2 (FPR2) [12]. Activation of this G protein-coupled receptor expressed on neutrophils, monocytes, macrophages, immature dendritic cells, and microglial cells induces chemotaxis, degranulation and superoxide generation [13]. While micromolar concentrations of PSMs are needed for neutrophil lysis, nanomolar concentrations are enough for FPR2-mediated neutrophil stimulation. Although neutrophils sense PSMs at nanomolar concentrations, *S. aureus* can evade FPR2 stimulation by producing the antagonists FPR2 inhibitory protein (FLIPr) [14] and its homologue FLIPr-like [15].

Most of the PSM activities are generally studied in normal culture medium without the addition of serum [9,12,16,17]. However, our group has recently discovered that PSMs are functionally inhibited by lipoproteins present in serum [18]. Lipoproteins perform a key role in the circulation by transporting lipids to and from the liver. Lipoproteins are complex particles with a neutral core containing triglycerides and cholesterol-esters, covered by an amphipathic monolayer of phospholipids and unesterified cholesterol and an additional apo-protein that binds and stabilizes the particles [19]. Reverse cholesterol transport is a pathway that describes the high density lipoprotein (HDL)-mediated removal of excess cholesterol from the peripheral cells to the liver for excretion by the bile [20,21,22]. Both lecithin cholesterol acyltransferase (LCAT) [23,24] and ATP-binding cassette transporter A1 (ABCA1) [21,25] play a central role in this process. ABCA1 is involved in the formation of lipid-poor apoA-I into pre-β-HDL, a discoidal particle that consists of phospholipids and apoA-I and stimulates the release of cholesterol. LCAT converts the cholesterol accepted by lipid-poor apoA-I into cholesteryl esters and transforms pre-β HDL into a small spherical HDL particle. ABCA1$^{-/-}$ mice have a total plasma HDL deficiency [26], whereas LCAT$^{-/-}$ mice only

have non-mature, cholesterol poor, pre-β-HDL particles in their plasma [27]. Although the evidence for the functional inhibition of PSMs by serum lipoproteins was obtained *in vitro, in vivo* PSM neutralization remains to be investigated. This study makes use of genetically targeted mice deficient for LCAT, ABCA, SR-BI and ApoE to evaluate the role of the various lipoproteins in *in vitro* neutrophil lysis assays. Furthermore, the contribution of HDL in PSM neutralization was investigated *in vivo* by monitoring neutrophil recruitment in a PSM-induced peritonitis model in LCAT$^{-/-}$ mice.

Materials and Methods

Ethics statement

Informed written consent was obtained from all donors and was provided in accordance with the Declaration of Helsinki. Approval was obtained from the medical ethics committees of the University Medical Center Utrecht (Utrecht, The Netherlands) and the Academic Medical Center (Amsterdam, The Netherlands).

Reagents

PSM peptides were synthesized with the published sequences [9] by Genscript at 95% purity. PSMα1 (MGIIAGIIKVIKSLIEQFTGK), PSMα2 (MGIIAGIIKFIKGLIEKFTGK), PSMα3 (MEFVAKLFKFFKDLLGKFLGNN), PSMα4 (MAIVGTIIKIIKAIIDIFAK), δ-toxin (MAQDIISTISDLVKWIIDTVNKFTKK) were all synthesized with an N-terminal formyl methionine residue. Peptide stocks were prepared at 2 mM and dissolved in H_2O except PSMα4, which was dissolved in 50% (v/v) MeOH/H_20.

Mice

SR-BI-deficient mice were kindly provided by Dr. Monty Krieger [31], LCAT$^{-/-}$ mice were a kind gift from Dr. Cheryl L. Wellington [45] , and ABCA1-deficient mice were kindly provided by Dr. G. Chimini [26]. Wildtype c57Bl/6 mice, homozygous LDL receptor$^{-/-}$ mice, originally generated by Ishibashi *et al*. [28] and apoE$^{-/-}$ mice were obtained from the Jackson Laboratory (Bar Harbor, ME) as mating pairs, and bred in Gorlaeus Laboratories (Leiden, The Netherlands). Mice were maintained on a sterilized regular chow diet, containing 4.3% (w/w) fat and no cholesterol (RM3, Special Diet Services, Witham, UK) or fed a Western-type diet (WTD) to induce atherosclerosis, containing 15% (w/w) fat and 0.25% (w/w) cholesterol (Diet W, Abdiets, Woerden, The Netherlands). Animal experiments were performed at the Gorlaeus Laboratories of the Leiden/Academic Center for Drug Research in accordance with the National Laws (ID 04081.1). For the peritonitis model 11-15 weeks old LCAT$^{-/-}$ and age matched C57BL/6 received an intraperitoneal injection with 1 mL of 10 μM or 30 μM PSMα3 or saline only. Six hours after PSMα3 exposure mice were euthanized and blood was drawn into EDTA through orbital extraction. The peritoneal cavity of the mice was lavaged with 10 mL cold PBS to collect peritoneal leukocytes. Leukocyte quantification of peritoneal lavage samples and blood was performed using an automated Sysmex XT-2000iV Veterinary Heamatology analyzer (Sysmex Corporation). Corresponding samples were cytospun for manual confirmation and cells were stained with Diff-Quick (Baxter) to visualize leukocyte accumulation in the peritoneum. Alternatively, peritoneal lavage leukocytes were lysed with 0.1% triton X-100 for myeloperoxidase (MPO) activity measurement as described [49]. Plasma was isolated by centrifugation of whole blood at 4000 rpm for 10 min at 4°C. All

experimental protocols were approved by the local ethics committee for animal experiments.

Cells
HL-60 cells stably transfected with the FPR2 (HL-60/FPR2), were kindly provided by F. Boulay (Laboratoire Biochimie et Biophysique des Systemes Integres, Grenoble, France). Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 2 µM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 600 µg/ml G418. Human neutrophils were isolated from venous heparinized blood by means of the Ficoll-Histopaque gradient method as described [50].

Plasma preparations
Freshly drawn venous blood from healthy controls as well as TD patients was collected after overnight fasting into 0.1 volume 130 mmol/L trisodium citrate. Plasma was obtained by centrifugation and stored until use at -70°C. Plasma was heat-inactivated by heating at 56°C for 20 min.

Biochemical Analyses
Murine plasma concentrations of free cholesterol, cholesteryl esters and total cholesterol were determined using enzymatic colorimetric assays. Absorbance was read at 490 nm. Precipath (standardized serum) was used as internal standard. The cholesterol distribution over the different lipoproteins in plasma was analyzed by fractionation of 50 µl pooled plasma of each mouse genotype using a Superose 6 column (3.2 x 30 mm; Smartsystem, Pharmacia). Free cholesterol and cholesteryl ester content of the effluent was determined using enzymatic colorimetric assays.
Total human plasma cholesterol was determined by an enzymatic colorimetric procedure (CHOD-PAP, Roche Diagnostics, Basel, Switzerland), HDL-cholesterol was measured as cholesterol remaining after precipitation of apoB-containing lipoproteins by $MnCl_2$. LDL-cholesterol was calculated using the Friedewald formula.

Calcium mobilization in human neutrophils and HL-60 cells
Calcium mobilization with isolated human neutrophils and HL-60/FPR2 cells was performed as previously described [18]. For this purpose, cells (5×10^6 cells/ml) were labeled with 2 µM Fluo-3-AM, protected from light, with gentle agitation, for 20 min at room temperature. The cells were washed and resuspended in RPMI-HSA (without FCS) to 5×10^6 cells/ml. Stimuli were prepared by incubating 25 µl of 10 times concentrated agonist with 25 µl 10 times concentrated heat inactivated human or mice plasma or buffer for 30 min at room temperature. Before stimulation, cells were diluted to 1×10^6 cells/ml in a volume of 200 µl. The background fluorescence level for Fluo-3 was monitored for 8 sec after which 50 µl of pre-incubated stimulus was added. The sample tube was rapidly placed back to the sample holder and the fluorescence measurement continued up to 52 sec. Cells were gated based on scatter parameters to exclude cell debris and the mean fluorescence value at basal level was subtracted from the value at peak level (at 30 sec). The different fluorescent values were expressed as percentage of the maximal response for each individual stimulus. Alternatively, various concentrations of culture supernatants or synthetic PSM, pre-incubated with various concentrations human or mice plasma for 15 min, were added to Fluo-3-labeled HL-60/FPR2 cells followed by flow cytometry.

Neutrophil lysis assay

Lysis of human neutrophils by synthetic PSMs was measured as described [18]. PSMs were pre-incubated with different concentrations of human or mice plasma for 10 min at room temperature. Pre-treated supernatants were transferred to a 96-wells microtiter plate (Nunc) containing 3×10^6 neutrophils in a total volume of 100 µl RPMI-HSA and incubated for 15 min at 37°C. Neutrophil lysis was determined by release of lactate dehydrogenase (LDH) using the CytoTox 96 Non-Radioactive Cytotoxicity kit (Promega) according to the manufactures protocols.

Statistics

Data were analyzed by Graph Pad Prism 5 software using two-tailed Student's t-test or a two-way ANOVA using Bonferoni posttest as appropriate. Probability values less than 0.05 were considered significant.

Results

Human and mouse plasma neutralize PSMs

Multiple functions for staphylococcal PSMs have been described, such as the ability to potently activate and attract neutrophils via FPR2 and induce neutrophil lysis. Recently, our group reported that the potency of PSMs to lyse or activate neutrophils is greatly reduced in the presence of body fluids, due to the presence of lipoproteins [18]. Here, we extended this line of investigation by examining the role of lipoproteins in neutralizing PSMs *in vivo* in mice. At first, we investigated whether PSMs are also inhibited by murine plasma. Therefore, we compared the effect of human and murine control plasma on the PSMα3-induced lysis of human neutrophils. Indeed, murine plasma inhibited the PSMα3-induced lysis of neutrophils as potently as human plasma (**Figure 1A**). Besides PSMα3, all other *S. aureus* PSMα peptides and δ-toxin were tested in this assay. Again, 1% murine plasma, equally effective as 1% human plasma, completely abrogated the lysis of human neutrophils induced by the different synthetic PSM peptides (**Figure 1B**). In addition to PSM-induced neutrophil lysis, we also investigated the effect of murine plasma on PSM-induced neutrophil activation via FPR2. Hereto, we used an FPR2-transfected HL-60 cell line (HL-60/FPR2) and examined the neutrophil-activating capacity of PSMα3 with and without addition of human or murine control plasma by measuring calcium mobilization (**Figure 1C**). Again, 0.1% human and murine plasma comparably reduced the activity of PSMα3. Moreover, all different α-type PSMs were significantly inhibited by murine plasma and as potent as human plasma (**Figure 1D**). Altogether, these data indicate that murine plasma; most probably plasma lipoproteins can functionally inhibit PSMs of *S. aureus*.

Plasma of genetically deficient mice with altered lipoprotein levels differently neutralize PSMs

To evaluate the relative contribution of the lipoprotein subsets in mice to PSMα3 neutralization, we performed experiments with plasma from genetically modified mice with varying concentrations of plasma lipoproteins, including plasma from low density lipoprotein (LDL) receptor (LDLr) $^{-/-}$ mice [28] and apoE$^{-/-}$ mice [29,30], two commonly

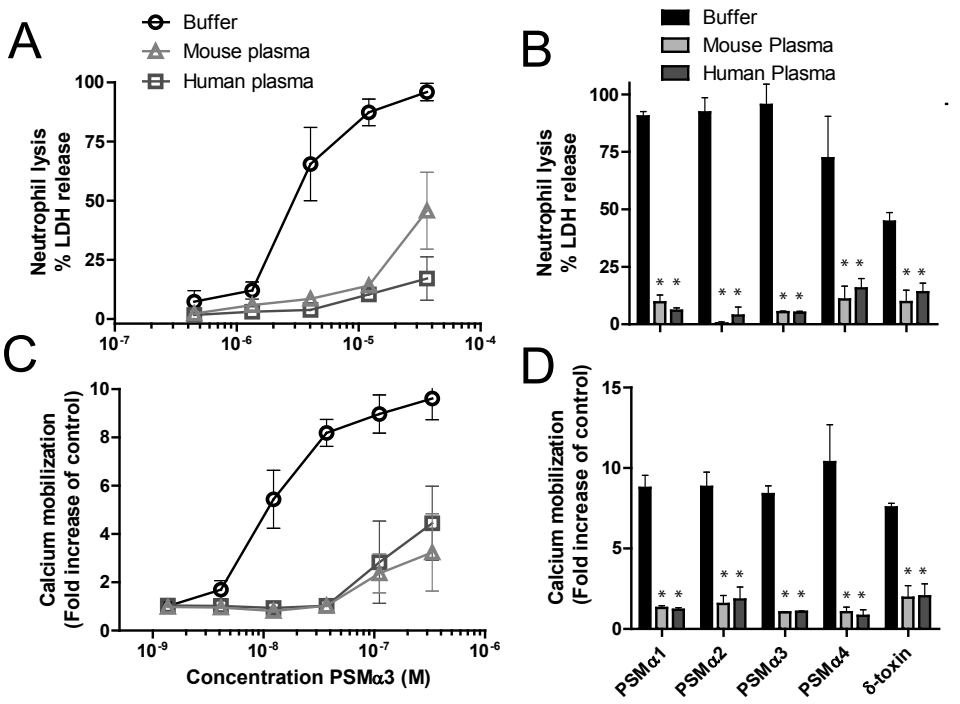

Figure 1. Human and murine control plasma inhibit PSM-mediated neutrophil lysis and activation. (A) Dose-dependent neutrophil lysis by synthetic PSMα3 (400 nM to 30 μM), preincubated with or without 1% human or murine control plasma. (B) Neutrophil lysis by synthetic PSMα1-3 (10 μM), PSMα4 and δ-toxin (50 μM) pretreated with or without 1% human or mouse plasma. Neutrophil lysis was measured through LDH release. (C) Dose-response curves for calcium mobilization in HL-60/FPR2 cells induced by PSMα3 with 0.1% human or mouse plasma-treated PSMα3. (D) Calcium mobilization of HL-60/FPR2 stimulated with 50 nM PSMα1, 500 nM PSMα2, 50 nM PSMα3, 5 μM PSMα4, and 200 nM δ-toxin, all pre-incubated with or without 0.1% human or mice plasma, before calcium mobilization was measured by flow cytometry. * Indicates statistical significance of $p<0.01$. Data represent means ± SEM of 3-5 independent experiments.

used hyperlipidemic mouse models. When fed regular chow diet (5.7% (w/w) fat and no added cholesterol), LDLr$^{-/-}$ mice show an elevation of cholesterol in the LDL fraction, which is further amplified after a high fat/high cholesterol Western-type diet (15% (w/w) cacao butter and 0.25% (w/w) cholesterol; WTD) challenge [28]. ApoE deficiency in mice already results in extremely elevated very low density lipoprotein (VLDL) and LDL cholesterol levels on regular chow diet [29,30]. In addition, we used the plasma of scavenger receptor BI (SR-BI)$^{-/-}$ mice, a mouse model with increased total plasma cholesterol levels mostly associated with abnormally large HDL particles [31] as well as plasma from ABCA1$^{-/-}$ [25] and LCAT$^{-/-}$ mice [32], two mouse models with reduced plasma HDL cholesterol levels. Plasma from these mice was evaluated for its effect on PSMα3-induced neutrophil lysis (**Figure 2A**). All types of plasma were capable of inhibiting lysis of neutrophils induced by 1 or 3 μM PSMα3. Similarly, plasma from LDLr$^{-/-}$ and apoE$^{-/-}$ mice maintained on regular chow diet had no impact on lysis induced by 10 μM PSMα3 compared to plasma from wildtype mice. In contrast, plasma from both LDLr$^{-/-}$ mice fed the high fat/high cholesterol WTD and SR-BI$^{-/-}$ mice significantly decreased PSMα-mediated neutrophil lysis. These results suggest that

elevated levels of LDL and HDL in plasma are responsible for the enhancement of PSMα3 neutralization.

To further evaluate the role of HDL, we tested the plasma from HDL deficient ABCA1$^{-/-}$ and LCAT$^{-/-}$ mice in the PSMα3-induced neutrophil lysis assay (**Figure 2B**). When PSMs were incubated with 0.5% WT plasma, neutrophils were protected against a challenge with a lethal dose of 1, 3 and 10 μM PSMα3, whereas incubation with plasma from LCAT$^{-/-}$ or ABCA1$^{-/-}$ mice failed to protect against lysis induced by 3 and 10 μM PSMα3. Again, this indicates that HDL is important in the protection against PSMα3-induced neutrophil lysis. This is in accordance with our previously published data on human lipoproteins, that showed that both HDL and LDL can sequester and neutralize PSMs, with HDL being the most potent lipoprotein in this process [18].

LCAT deficiency enhances neutrophil influx in a murine PSM-induced peritonitis model

PSMs have the capacity to recruit neutrophils in a mouse air-pouch model [12]. To investigate whether PSMα3 neutralization by HDL occurs *in vivo,* we used a PSMα3-induced peritonitis model and monitored peritoneal neutrophil recruitment in WT versus LCAT$^{-/-}$ mice. Basal

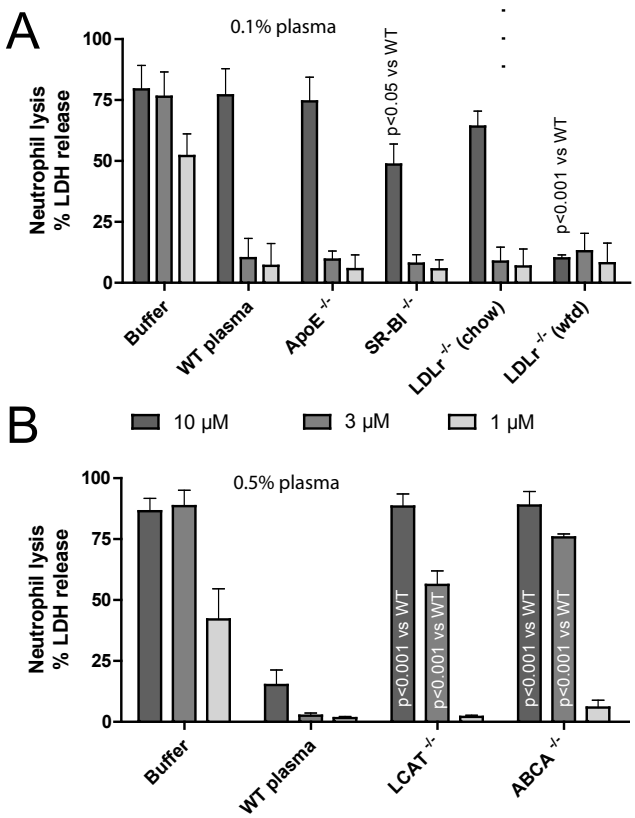

Figure 2. Altered protection of PSM-mediated neutrophil lysis by preincubation with plasma from WT, apoE$^{-/-}$, SR-BI$^{-/-}$, LDLr$^{-/-}$, ABCA1$^{-/-}$ or LCAT$^{-/-}$ mice. Dose-dependent human neutrophil lysis by synthetic PSMα3 (10, 3 and 1 μM), preincubated with 0.1 % plasma from (A) WT mice, apoE$^{-/-}$, SR-BI$^{-/-}$ and from LDLr$^{-/-}$ on normal (chow) and western type diet (WTD), (B) or pretreated with 0.5% plasma from WT, ABCA1$^{-/-}$ or LCAT$^{-/-}$ mice. Neutrophil lysis was measured through LDH release. Data represent means ± SEM of at least 3 independent experiments.

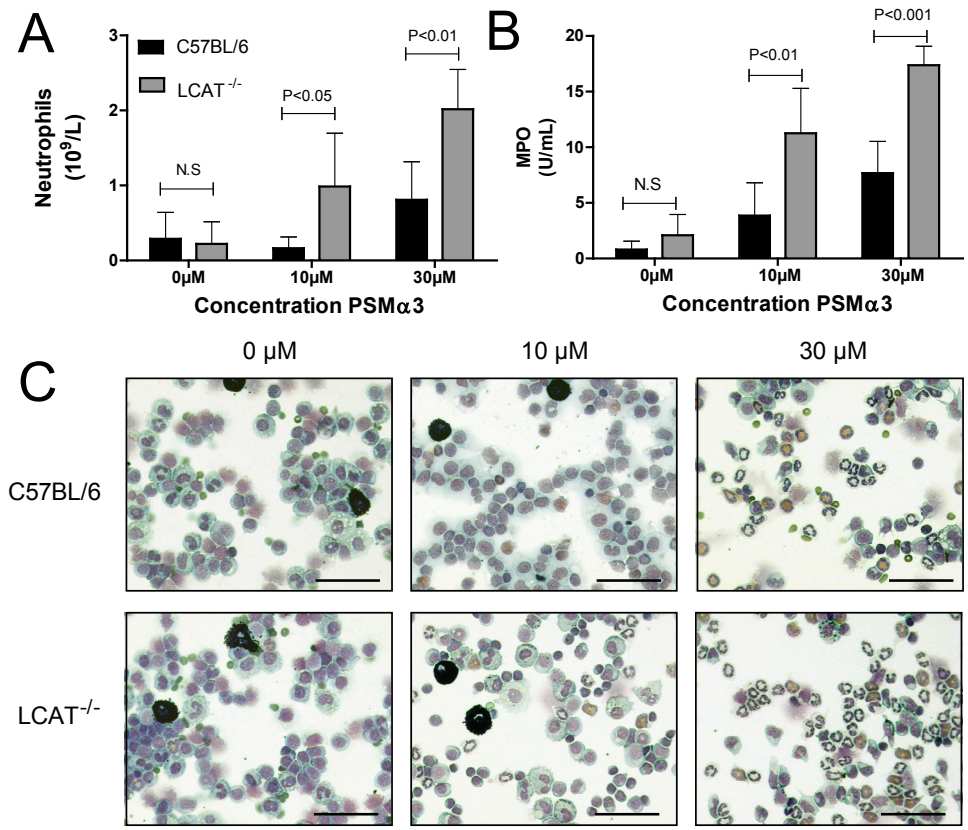

Figure 3. LCAT deficiency enhances neutrophil influx in murine PSM-induced peritonitis model. Peritonitis was induced by injecting 1 mL of saline or 10 μM or 30 μM filter sterilized PSMα3. Peritoneal leukocytes were harvested and analyzed 6 hours after PSM injection, using a hematology Sysmex XT-2000iV analyzer and the number of neutrophils was quantified (A) or MPO activity of lysed peritoneal cells was determined (B). Values represent the mean ± SD of 3-4 mice per group. (C) Photomicrographs of representative diff-quick stained cytospins of peritoneal cells. Original magnification ×200, bars indicate 50 μm.

leukocyte levels were determined by monitoring the leukocyte content of saline injected mice. The total basal number of leukocytes was similar between LCAT$^{-/-}$ (n=3) and WT mice (n=5) (data not shown). Interestingly, upon challenge of the mice with either 10 μM or 30 μM of PSMα3, the peritoneal neutrophil recruitment in LCAT$^{-/-}$ mice was significantly enhanced (**Figure 3A-B**). This enhanced neutrophil influx is also seen in cytospin preparations from the peritoneal lavage fluid, as more ring-shaped nucleated cells are present in the peritoneal lavage of PSMα3-treated LCAT$^{-/-}$ mice (**Figure 3C**). Together, these data indicate that HDL protects against PSMα3-induced inflammation in mice.

Plasma from patients with impaired ABCA1 function do not neutralize PSMs

The importance of ABCA1 in reverse cholesterol transport is evident in individuals harboring defects in their ABCA1 gene. These individuals suffer from a very rare disorder, called Tangier disease (TD), which is characterized by clinical hallmarks, including enlarged

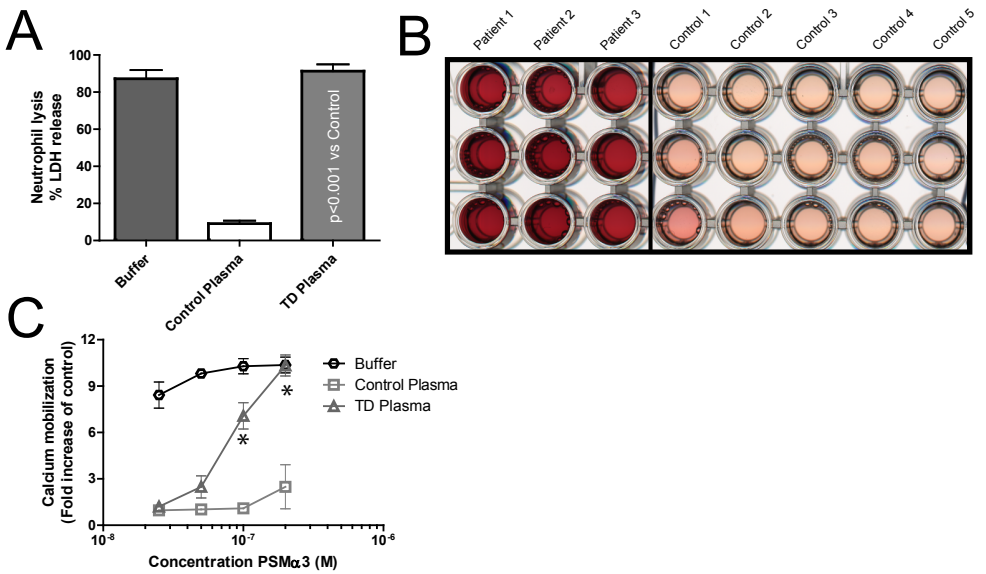

Figure 4. Plasma from Tangier patients is unable to protect against PSM-induced neutrophil activation and lysis. (A) PSMα3 (10 µM) induced human neutrophil lysis after preincubation with 0.5% plasma from three different Tangier disease (TD) patients and five healthy control donors. Neutrophil lysis was measured through LDH release. (B) Representative photograph of 1 out of 3 neutrophil lysis experiments, as explained under A. (C) Calcium mobilization of HL-60/FPR2 stimulated with (25 – 200 nm PSMα3) with and without pretreatment with 0.01% Tangier disease (TD) plasma or control plasma. Calcium mobilization was measured by flow cytometry. * Indicate statistical significance of p<0.01. Data represent means +/- SEM of 3 independent experiments.

lipid-laden tonsils and low plasma HDL [33]. Therefore, we investigated whether plasma of these patients could similarly decrease the potential to protect against staphylococcal PSMs. Indeed, plasma from 3 TD patients did not protect against PSMα3-induced neutrophil lysis *in vitro*, in contrast to control plasmas (**Figure 4A-B**). In addition, plasma of TD patients was significantly less effective in neutralization of PSMα3-mediated calcium fluxes in FPR2-HL60 cells. Thus, HDL deficient plasma of TD patients fails to protect against PSM-mediated neutrophil activation and lysis.

Discussion

The number of infections by *S. aureus* is increasing, especially infections caused by methicillin-resistant *S. aureus* (MRSA) strains [34]. Nevertheless, infections occur only in a small percentage of colonized individuals, which means that the innate immune system efficiently controls staphylococcal infections. In recent years it has become clear that besides the well-known players, such as immune cells, complement, antimicrobial peptides and chelating proteins, lipids and lipoproteins participate in innate immunity against invading microorganisms as well. Numerous studies have shown that lipoproteins bind microbes or cell-wall components derived from microorganisms [35]. For instance, lipoteichoic acid (LTA) present in the cell wall of staphylococci, or lipopolysaccharide (LPS), the major

outer membrane component of Gram-negative bacteria, bind mainly to HDL within human blood [36,37]. This binding to HDL neutralizes the pro-inflammatory effects of LPS or LTA, inhibiting their interaction with Toll-like receptors and activation of immune cells [38]. Furthermore, apolipoprotein B-containing lipoproteins hamper the communication between staphylococcal cells [39]. Accumulation of secreted autoinducing peptide (AIP) is normally sensed by the accessory gene regulator (*agr*) system and triggers the secretion of toxins and extracellular enzymes. However, apoB in VLDL and LDL present in the blood can sequester AIP and thereby disable staphylococcal quorum sensing. Moreover, mice lacking plasma apoB are more susceptible to invasive staphylococcal infections, highlighting that apoB is a crucial innate defense effector against *S. aureus* [39]. Recently, we reported that HDL and LDL present in the blood specifically inhibit staphylococcal PSMs. When human serum was spiked with PSMs and different lipoprotein subsets were analyzed for the presence of PSMs, approximately 80% of the spiked PSMs were found in the HDL-containing fractions, whereas approximately 15% and 5% of PSM was recovered in LDL- and VLDL-containing fractions, respectively. Thus, within human blood, HDL is the most potent inhibitor of PSMs [18].

In the current study, we used plasma from genetically targeted mice to pinpoint the relative contributions of the lipoprotein subsets. Plasma from SR-BI$^{-/-}$ mice on normal diet and LDLr$^{-/-}$ mice on WTD showed enhanced protection from PSM-induced lysis of human neutrophils, whereas plasma from apoE$^{-/-}$ and LDLr$^{-/-}$ mice on regular chow diet gave no additive protection. These results show that subtle changes in HDL levels in SR-BI mice enhances protection, whereas only highly elevated LDL levels in LDLr$^{-/-}$ plasma are able to enhance protection. HDL deficiency in LCAT$^{-/-}$ and ABCA1$^{-/-}$ mice resulted in a dramatic reduction in the protection against PSM-induced neutrophil lysis, again pinpointing to HDL as a major lipoprotein responsible for PSM neutralization.

This study addressis the important question whether interstitial lipoprotein levels are sufficient for PSM neutralization. PSM-induced peritonitis in HDL deficient LCAT$^{-/-}$ mice resulted in enhanced neutrophil influx, showing that *in vivo* neutralization of PSMs occurs by HDL particles. This implicates that tissue lipoprotein levels are sufficient for uptake of PSMs and thereby dampening of the host immune response. In line with this, Sigel *et al.* showed an enhanced pro-inflammatory cytokine response after *S. aureus* challenge in mice where lipoprotein secretion was pharmacologically inhibited [40]. It is tempting to speculate that the lack of PSM neutralization due to the low lipoprotein levels contributed to these findings. Nevertheless, experimental infection models with *S. aureus* still have to be performed in LCAT$^{-/-}$ mice to determine the exact contribution of HDL-dependent PSM neutralization in staphylococcal pathogenesis.

Finally, plasma of Tangier disease patients with impaired ABCA1 function, thereby lacking HDL, was not able to protect against PSM-mediated functions. The population of Tangier disease patients is too small to perform robust epidemiology, however HDL-deficiency might be a predisposing risk factor for staphylococcal disease within this patient group.

PSMs can lyse neutrophils and *S. aureus* can produce PSMs in very large quantities [8,9]. However, the majority of these PSMs will rapidly be neutralized by serum lipoproteins, making it unlikely that these high concentrations required for neutrophil lysis, can be reached *in vivo* [18]. However, PSMs could function within neutrophils after phagocytosis of *S. aureus.* Intracellular lysis could account for the fact that studies with *psm* knockout *S. aureus* strains show reduced virulence in mice and rabbits [9,11]. Therefore, we think that

PSMs function mainly as intracellular toxins in an environment where no lipoproteins are present. Multiple lines of evidence have accumulated in recent years, demonstrating that *S. aureus* can survive within cells, or even use host cells as transport to disseminate from the site of infection [41,42,43]. However, to escape from the phagosome, staphylococci need to adopt a more virulent phenotype [44]. This transition is dependent on the intracellular activation of the *agr* system [45]. The concentration of the quorum sensing pheromone AIP can reach the critical concentration necessary for *agr* activation inside host cells [46,47]. In accordance with these studies, we have shown with PSM-GFP reporter constructs that the *psmα* operon is activated after phagocytosis of *S. aureus* by neutrophils [18]. Very recently, it was demonstrated that the stringent response, characterized by the rapid synthesis of (p)ppGpp as messenger of environmental stress conditions, seems to precede the quorum sensing mechanism. Both systems appear to be crucial for up-regulating PSMs in the neutrophil phagosome and thereby contribute to the lysis of neutrophils after phagocytosis, enabling the subsequent escape of the staphylococci [48]. However, among different *S. aureus* strains several mechanisms, instead of a single virulence factor, play a role in staphylococcal escape after phagocytosis; and different toxins may cause escape in different cell types.

Overall, the current study shows that HDL, an important plasma component for lipid metabolism, is able to functionally neutralize staphylococcal PSMs. These findings emphasize another important role of HDL in host defense by functioning as a sink for extracellular PSMs.

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5

Staphylococcal α-PSMs Contibute to Neutrophil Lysis after Phagocytosis

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Abstract

Staphylococcus aureus community-acquired (CA) MRSA strains are highly virulent and can cause infections in otherwise healthy individuals. The most important mechanism of the host for clearing *S. aureus* is phagocytosis by neutrophils and subsequent killing of the pathogen. Especially CA-MRSA strains are very efficient in circumventing this neutrophil killing. Interestingly, only a relative small number of virulence factors have been associated with CA-MRSA, one of which are the phenol soluble modulins (PSMs). We have recently shown that the PSMs are functionally inhibited by serum lipoproteins, indicating that PSMs may exert their cytolytic function primarily in the intracellular environment. To further investigate the intracellular role of the PSMs we measured the effect of the α-type and β-type PSMs on neutrophil killing after phagocytosis. Using fluorescently labeled *S. aureus*, we measured bacterial survival after phagocytosis in a plate reader, which was employed next to flow cytometry and time-lapse microscopy. Phagocytosis of the CA-MRSA strain MW2 by human neutrophils resulted in rapid host cell death. Using mutant strains of MW2, we demonstrated that in the presence of serum, the intracellular expression of only the *psm*α operon is both necessary and sufficient for both increased neutrophil cell death and increased survival of *S. aureus*. Our results identify PSMα peptides as prominent contributors to killing of neutrophils after phagocytosis, a finding with major implications for our understanding of *S. aureus* pathogenesis and strategies for *S. aureus* vaccine development.

Introduction

Staphylococcus aureus (S. aureus) has the ability to cause many skin and soft tissue infections and more serious invasive diseases such as sepsis, endocarditis, osteomyelitis, toxic shock syndrome and pneumonia [1]. Moreover, these infections are becoming increasingly difficult to treat due to the acquisition of antibiotic resistance [2]. In recent years, there has been an emergence of community-associated (CA)-MRSA strains that cause infections outside of the healthcare setting in otherwise healthy individuals. This capacity is believed to be due to the fact that CA-MRSA strains are more virulent than hospital-associated MRSA strains [3,4]. This enhanced virulence potential is in part related to the ability of these strains to evade or kill human neutrophils, the most prominent cellular innate host defense against invading microorganisms [5]. Neutrophils take up pathogens by phagocytosis, which in turn triggers the production of reactive oxygen species (ROS) and the release of microbicidal granule components into the forming phagosomes. These responses are sufficient for killing most pathogens; however, *S. aureus* are to some extend resistant to phagocytic killing in part and cause human infections. CA-MRSA strains seem to be very efficient in circumvention of neutrophil killing [6,7].

For full virulence, *S. aureus* can make use of a myriad of toxins. However, only a small number of toxins have been associated with the enhanced virulence of CA-MRSA [5]. Among these are the phenol soluble modulins (PSMs). PSMs are small amphipathic α-helical peptides of approximately 20-25 (α-type) and 44 (β-type) amino acids [8]. Due to their amphipatic helical structure PSMs can facilitate lysis of neutrophils, peripheral blood mononuclear cells, and erythrocytes, most likely through a receptor-independent process [8,9]. Only α-type PSMs have cytolytic activities and it is not yet understood why β-type PSMs lack such properties. In *S. aureus*, the α-type PSM group consists of four PSMα peptides and the δ-toxin [8]. Neutrophils can recognize PSMs via their FPR2 receptor, which results in activation and chemotactic attraction [10,11]. Although the PSM-encoding loci are conserved in the core genome of all sequenced staphylococcal strains, the *in vitro* expression correlates with the more virulent phenotype between CA-MRSA and HA-MRSA [8,12]. The production of PSMs is strictly regulated by the accessory gene regulator (*agr*), the global regulatory/quorum sensing system, although independent of RNAIII [13]. Multiple animal models have shown a role of PSMα peptides in experimental infection. Isogenic *psm*α mutant strains have significantly reduced virulence in bacteremia and skin infection models compared to their wild-type CA-MRSA strains [8,14].

Recently, our group has discovered that PSMs are functionally inhibited by serum lipoproteins [15]. These lipoproteins are abundantly present within the blood and human tissue, indicating that PSMs exert their function primarily in the intracellular environment. Thus, we hypothesized that there is an important function for PSMs as intracellular toxins and here sought to determine the role of PSMs after phagocytosis.

Materials and Methods

Ethics statement

Informed written consent was obtained from all donors and was provided in accordance

with the Declaration of Helsinki. Approval was obtained from the medical ethics committee of the University Medical Center Utrecht (Utrecht, The Netherlands).

Bacterial strains and culture conditions

The CA-MRSA strain MW2 [45] and isogenic *lukSF, psm* and *agr* deletion mutants [8,46] were used for all experiments. In the *Δhld s*train, translation of *hld* is interrupted by a single base mutation of the start codon, which allows for maintained function of RNAIII. The *psmα* gene locus was complemented in *Δα* strain as described [8]. The MW2 strain and all isogenic *psm* mutant strains were transformed with pCM29 [39] or pTH3 (Nijland *et al*, in prep.) that from the *sarA*P1 promoter constitutively and robustly express the superfolder green fluorescent protein (sGFP) or the cyan fluorescent protein Cerulean (CFP) respectively. Additionally, the Δαβ*hld* strain was transformed with pCM29 expressing cyan fluorescent protein (CFP). Bacteria were grown in Müller-Hinton broth (MHb) at 37°C while shaking. When appropriate, tetracycline (12.5 μg/mL) or chloramphenicol (10 μg/mL) was added for o/n maintenance of the plasmids.

***Ex vivo* phagocytosis assay**

Neutrophils were isolated as previously described [15]. To investigate the *S. aureus*-mediated killing of human neutrophils after phagocytosis, MW2, the isogenic *psm*, *lukSF* and *agr* mutants or similar strains constitutively expressing GFP were used. Bacterial strains were grown o/n in MHb supplemented with 10 μg/mL chloramphenicol. On the day of the experiment, the strains were diluted 20x in MHb without antibiotics and cultured until late log phase (OD_{660nm} 1.0). Bacteria were washed and resuspended in RPMI-1640 containing 25 mM HEPES, L-glutamine (Biowhittaker), and 0,05% human serum albumin (HSA; Sanquin) (RPMI-HSA). Freshly isolated neutrophils were pre-treated with or without 10 μg/mL FLIPr-Like (FcγR-inhibitor) or with and without cytochalasin D (Sigma) to investigate the influence of phagocytosis. Subsequently, $5x10^5$ neutrophils were mixed with $5x10^6$ bacteria (MOI=10) in the presence of 10% normal human pooled serum (15 donors) or heat-inactivated serum (from same serum pool) in a final volume of 100 μL RPMI-HSA. Phagocytosis was initiated at 37°C with shaking for 15 min. For some experiments phagocytosis was measured by flow cytometry. Neutrophils were gated based on their forward and side scatter profiles and the percentage of neutrophil-associated GFP-positive bacteria was used as measurement for phagocytosis. In these conditions both ingested bacteria as well as bacteria bound to the neutrophil surface are measured. We will refer to both these phenomenons as phagocytosis. For other experiments, phagocytosis was followed by various incubation times at 37°C while gently shaking. Cells were pelleted by centrifugation at different time points after infection and neutrophil lysis was determined in the supernatant by detection of lactate dehydrogenase (LDH) using the CytoTox 96 Non-Radioactive Cytotoxicity kit (Promega), according to the manufacturer's protocol.

Staphylococcal outgrowth after phagocytosis

Neutrophil-mediated killing of *S. aureus* and staphylococcal outgrowth after phagocytosis was monitored by culturing and phagocytosis of the staphylococcal strains, as described above, with the exception that neutrophils were seeded at $2.5x10^5$ cells per well and infected with $2.5x10^6$ bacteria (MOI=10). At different time points post phagocytosis the neutrophils were lysed by 10 times dilution in 0.1% Saponin H20 (pH 11.0) for 15 min on

ice. Bacteria were than serially diluted in 10 fold dilutions in PBS and plated on sheep blood agar plates (Oxoid). Recovered bacteria were determined by counting CFUs and normalized at each time point to the input CFU. Alternatively, bacterial outgrowth was measured as GFP fluorescence. After initiation of phagocytosis, neutrophils were transferred to a clear 96 well flat bottom polystyrene tissue culture plate (Greiner) and 50 µl RPMI-HSA was added, resulting in 150 µL culture/well. The plate was grown in a Fluostar Omega plate reader (BMG labtech) at 37°C with constant double orbital shaking (400 rpm) in between measurements. Both the absorbance at 660 nm and GFP fluorescence (excitation 485 nm/emission 520 nm) were measured every 10 minutes for each well. The signal from 4 identical wells was averaged and corrected for blank wells containing only medium.

Time-lapse microscopy

Each well of a 8 well Lab-Tek II chambered coverglass (Thermo Scientific) was loaded with 200 µl of RPMI-HSA. $1x10^5$ neutrophils that had phagocytosed GFP-labeled MW2 strains as described above were added and were allowed to settle at the bottom of the well. Propidium iodine (Invitrogen) was added at a final concentration of 4 µM to visualize disruption of neutrophil membranes in time. Microscopic image acquisition of neutrophils and bacteria was performed using a Leica TSC SP5 inverted microscope equipped with a HCX PL APO 40 x 0.85 objective (Leica Microsystems, The Netherlands). The microscope was encased in a dark environment chamber that was maintained at 37°C. The cells and bacteria were monitored for CFP (CFP ET filter cube) and/or GFP (GFP ET filter cube), PI (N21 filter cube), and brightfield every 7 minutes at four positions in each well. To create a time lapse movie of the interaction between the neutrophils and the MW2 strains, the separate channels were combined and rendered as a time-lapse movie using Leica LAS AF software.

Statistics

Statistical analysis was performed using Graph Pad Prism Version 5 with two-tailed unpaired T-tests (two groups) or one-way ANOVA with Bonferoni posttest (multiple groups).

Results

Phagocytosis and subsequent killing of the pathogen is the most important mechanism of bacterial clearance by the host [5]. Here, we used *S. aureus* strain MW2 as a model CA-MRSA strain and investigated whether this strain could resist the killing mechanisms employed by human neutrophils after phagocytosis. To measure phagocytosis, freshly isolated human neutrophils were incubated in the presence of 10% human serum with a derivative of strain MW2, which constitutively expresses GFP (MW2-GFP). Complement and immunoglobulins (Ig) present in the serum will opsonize the bacterium [5]. Additionally, generated C5a and bacterial factors will prime the neutrophils, resulting in phagocytic uptake of the bacteria. To validate our bacterial killing assays, phagocytosis was blocked in two ways. First, heat-inactivation (HI) of serum at 56°C inactivated complement protein C2 and Factor B, thereby completely blocking opsonization of the bacterium by complement [16]. Additionally, formyl peptide receptor inhibitory protein-like (FLIPr-Like) protein was used to block Fcγ-receptors and prevent Ig-mediated phagocytosis [17]. Fcγ-receptors recognize Igs and, together with complement receptors (CR), constitute the most important phagocytic receptors on human

phagocytes. Secondly, cytochalasin D (CytD) was used to inhibit phagocytosis by blocking actin polymerization.

Phagocytosis of *S. aureus* MW2 induces rapid neutrophil cell-death

In the presence of human serum, neutrophils can efficiently phagocytose MW2-GFP, as demonstrated by measuring GFP fluorescence associated with neutrophils by flow cytometry (**Figure 1A**). At a multiplicity of infection (MOI) of 10, 96.8% of the neutrophils have phagocytosed at least one GFP-expressing bacterium and the bacterial uptake is nearly complete. Heat-inactivation of the serum in combination with FLIPr-Like treatment of neutrophils nearly completely inhibited phagocytosis, whereas CytD treatment of neutrophils used in several studies as potent inhibitor of phagocytosis only partly inhibits phagocytosis. One explanation could be that opsonized bacteria can still associate with neutrophils via CR or Fcγ-receptors, but cannot be ingested, since this process involves the

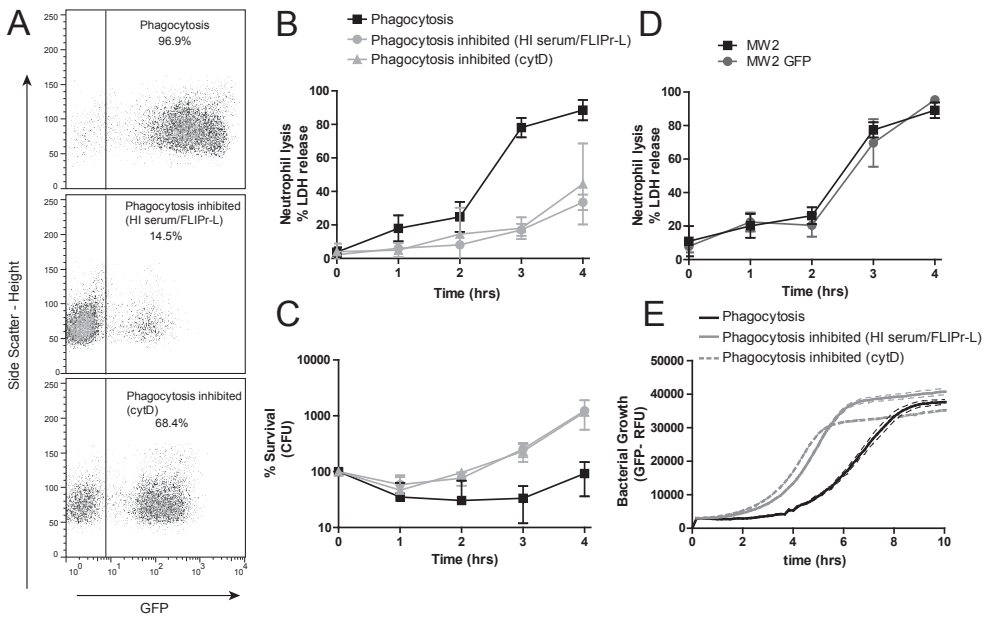

Figure 1. Impact of phagocytosis of S. aureus MW2 on neutrophil lysis and bacterial survival. (A) Analysis of neutrophil phagocytosis of CA-MRSA MW2 by flow cytometry. Neutrophils were allowed to phagocytose staphylococcal strain MW2, which constitutively expresses GFP (MW2-GFP), in the presence of human serum for 30 min. Phagocytosis was blocked by either heat-inactivation (HI) of the serum combined with pre-incubation of neutrophils with Flipr-Like (FcγR-inhibitor) or by addition of cytochalasin D. One representative scatterplot out of three independent experiments is shown. (B) Time dependent lysis of human neutrophils after phagocytosis of MW2 (upper panel) and bacterial survival (lower panel). Neutrophils were mixed with pre-cultured MW2 in the presence of human serum, allowing phagocytosis, or phagocytosis was blocked, as described above. Neutrophil lysis was measured through LDH release. (C) Bacterial survival was measured by counting CFUs. Data represent means ± SEM of 4-5 independent donors. (D) Time dependent lysis of human neutrophils after phagocytosis of MW2 or MW2-GFP as described above. (E) Impact of phagocytosis on bacterial rebound measured by GFP fluorescence every 5 minutes for 10 hours. Measurement started 30 min after initiation of phagocytosis. Phagocytosis was established as described above. Data represent the mean ± 95% coincidence intervals out of 3 independent experiments.

polymerization of actin.

Next, we used the wild type, non-fluorescent, MW2 strain for phagocytosis assays with human neutrophils at the same MOI of 10 and evaluated lysis of neutrophils and killing of bacteria at different time points after infection. Neutrophil lysis was determined by measuring LDH release, whereas for bacterial killing growth colony forming units (CFUs) were counted for each time point. Neutrophil phagocytosis of MW2 induced rapid neutrophil cell death (**Figure 1B**), which is in line with previously published data [6,7]. In contrast, blocking phagocytosis by HI-serum+FLIPr-Like or CytD treatment, resulted in a considerably delayed destruction of neutrophils by MW2. Although phagocytosis resulted in killing of MW2 at early time points, there was a rebound of bacterial growth starting 4 hours after phagocytosis (and at which point there is significant lysis of neutrophils), indicating survival of bacteria after neutrophil phagocytosis (**Figure 1C**; black line). When phagocytosis was blocked by HI-serum+FLIPr-Like or CytD treatment, bacterial growth started 2 hours earlier, as compared to bacteria that underwent phagocytosis (**Figure 1C**; grey lines). Moreover, there was a clear correlation between the lysis of neutrophils and the survival of MW2 (**Figure 1B**).

Measuring bacterial survival using GFP

These bacterial growth results were used to validate an alternative to the labor-intensive counting of CFUs, measuring bacterial survival and outgrowth by GFP-fluorescence produced by living MW2-GFP bacteria in a fluorescence plate reader. Using fluorescence, we were able to measure bacterial outgrowth after phagocytosis, where absorbance measurements were not possible, due to the interference of neutrophils in the culture. No difference in neutrophil lysis could be observed when cells were infected with wild type MW2 or MW2-GFP *S. aureus* (**Figure 1D**), nor did these bacteria grow differently in culture medium (data not shown). To determine the effect of phagocytosis on the growth of MW2-GFP, the same experimental setup was used as before, allowing phagocytosis in the presence of serum or blocking phagocytosis by HI-serum+FLIPr-Like or CytD treatment. The only difference was the measurement of MW2-GFP growth, which was performed in a fluorescent plate reader, instead of counting CFUs. Comparable to the results shown in **Figure 1C,** blockade of phagocytosis resulted in earlier outgrowth of MW2-GFP, whereas phagocytic killing resulted in a clear delay in bacterial outgrowth, measured as GFP fluorescence (**Figure 1E**). Of note, adding no neutrophils or only buffer without opsonins resulted in early outgrowth, similar to blocking phagocytosis, demonstrating only minor extracellular killing of MW2-GFP by neutrophils in these assay conditions (data not shown). Overall, in accordance with previous findings, we found that phagocytosis of *S. aureus* strain MW2 by neutrophils results in killing of the bacteria using our assay conditions. However, some bacteria can survive in these *in vitro* conditions and over time escape from neutrophils and replicate.

Time lapse fluorescent microscopy was used to visualize neutrophil lysis and bacterial growth after MW2-GFP phagocytosis on a single cell basis. MW2-GFP was incubated with neutrophils as described above to allow for phagocytosis. Both the growth of the bacteria (GFP) and the disruption of the neutrophil membranes (PI, red staining) were monitored over time. The results obtained using this setup were similar compared to those obtained in the experiments described above. When no phagocytosis takes place, the bacteria are able to rapidly replicate in the growth medium, and eventually most neutrophils become

PI positive. However, when MW2-GFP is phagocytosed, bacterial growth is clearly delayed, but neutrophil lysis is advanced (**Figure 2** and **Supplementary movies 1-4**), illustrating that neutrophil phagocytosis of staphylococci promotes lysis of human neutrophils. Moreover, a certain percentage of *S. aureus* MW2-GFP escapes after phagocytosis by neutrophils and replicate in the extracellular milieu.

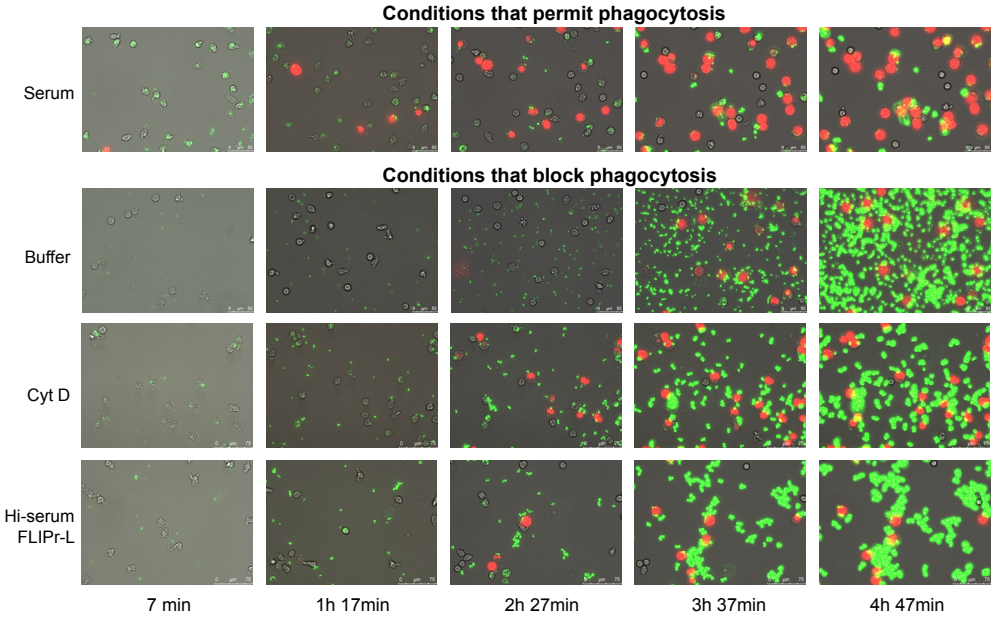

Figure 2. Time lapse analysis of neutrophil lysis and bacterial outgrowth. Analysis of neutrophil phagocytosis of CA-MRSA strain MW2 by time lapse fluorescence microscopy. Neutrophils were incubated with strain MW2, which constitutively expresses GFP (MW2-GFP) for 30 min, in the presence of human serum (phagocytosis), or absence of opsonin (Buffer) (No phagocytosis), or serum with addition of cytochalasin D (CytD), or heat-inactivated serum together with pre-incubation of neutrophils with FLIPR-Like (FcγR-inhibitor) (HI-HPS+Flipr-L). A representative frame series out of three independent experiments is shown. Brightfield (grey), GFP (green) and PI (red) channels are combined.

PSMα peptides produced inside neutrophils promote lysis of neutrophils and survival of *S. aureus*

Many studies have suggested a prominent role for PSMs in staphylococcal virulence, based on their capacity to lyse and activate neutrophils [8]. However, as we described before, in the presence of serum lipoproteins PSMs may be functionally neutralized [15]. We reasoned that PSMs could function after phagocytosis and promote neutrophil lysis from inside the phagosome. To address this possibility, neutrophils were infected with MW2 and MW2 derivatives, in which single or all *psm* operons, or the *agr* system were deleted (Δ*agr*, Δα, Δβ,Δ*hld*,Δαβ*hld*). In addition, we included a PVL-negative mutant of strain MW2 (MW2Δ*lukSF*) in our experiments to compare the effects of PSMs on intracellular lysis to PVL. Furthermore, a PSMα-complemented Δα pTXα strain was tested. First, we investigated whether the phagocytic uptake is similar for all strains. Therefore, we fluorescently labeled these strains and measured phagocytosis 30 min after infection. No difference in the rate of phagocytosis was observed, demonstrating that all strains were equally well ingested

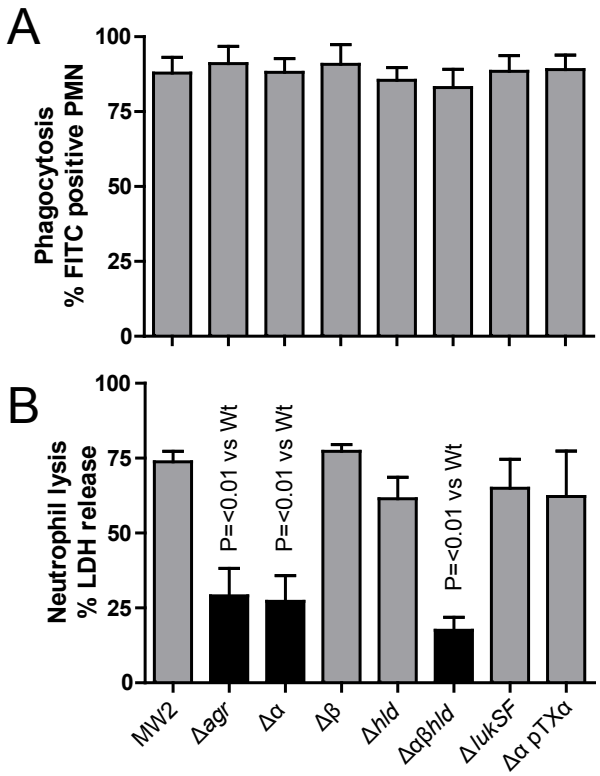

Figure 3. Impact of PSM deletions on neutrophil lysis after phagocytosis. (A) Analysis of neutrophil phagocytosis of FITC-labeled MW2 and isogenic *agr*, *psm*, and *LukSF* mutant strains. Neutrophils were allowed to phagocytose MW2 strains in the presence of human serum. After 30 min phagocytosis was measured by flow cytometry. (B) Neutrophil lysis 3hrs after phagocytosis (MOI 10) of MW2 strains, as determined by LDH release. Data represent the mean ± SEM of 4-5 independent experiments with neutrophils isolated from different donors.

by human neutrophils (**Figure 3A**). Three hours after infection, lysis of neutrophils was monitored by LDH release. The production of many virulence toxins are under control of the *agr* system, including the PSMs. Significantly less neutrophils were lysed when cultured with the Δ*agr* strain (**Figure 3B**), although not to the extent of those lysed by the wild-type strain, indicating mechanisms of *agr*-independent neutrophil lysis exist. Interestingly, the *psm*α operon is fully responsible for this effect, as the neutrophil lysis caused by Δα and Δαβ*hld* strains was comparable to that caused by the Δ*agr* strain. In addition, with the *psm*α-complemented Δα pTXα strain recovery of the wild type MW2 phenotype was accomplished. Other PSM loci and PVL are not important for neutrophil lysis after phagocytosis, as they did not influence the neutrophil lysis compared to wild type MW2. Taken together, these date indicate that PSMα peptides produced by intracellular *S. aureus* promote lysis of human neutrophils.

Next, we investigated whether the observed decrease in neutrophil lysis when using the MW2Δα and Δαβ*hld* strains impacts bacterial survival and subsequent outgrowth. For this purpose, neutrophils were fed MW2, Δα, Δβ,Δ*hld*, and Δαβ*hld* strains, which constitutively express GFP on a plasmid. Subsequently, GFP fluorescence was monitored in a fluorescence

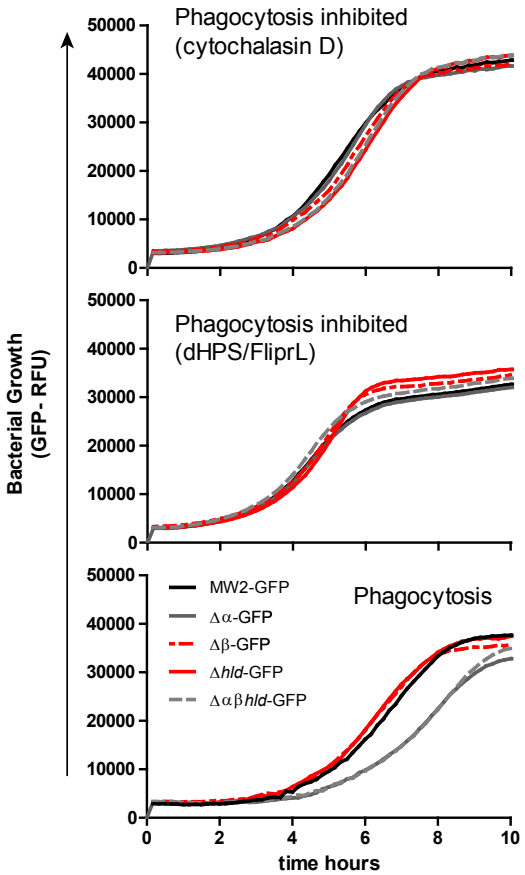

Figure 4. Staphylococcal outgrowth after phagocytosis. Neutrophils were allowed to phagocytose MW2, Δα, Δβ,Δ*hld*,Δαβ*hld* strains, which are transformed to constitutively express GFP (MOI 10). Alternatively, phagocytosis was blocked by HI-serum and FcγR-inhibitor FLIPr-Like or by 20 µg/mL Cytochalasin D. Bacterial growth represented by GFP Fluorescence was monitored for 10 hours after infection. Results represent the means out of 4 replicates.

plate reader for 10 hours to investigate bacterial growth. When phagocytosis was blocked, no difference in bacterial growth was observed between the different mutants (**Figure 4**). When phagocytosis occurred, there was a clear delay in outgrowth of the Δα and Δαβ*hld* strains, whereas the growth curves of the other *psm* mutant strains were indistinguishable from that of the wild type MW2 strain. Previously we and others have shown that PSM expression is upregulated after phagocytosis of staphylococci [15,18]. These results further confirm that PSMα peptides produced in the intracellular space promote the escape of *S. aureus* from the neutrophil.

Time lapse video microscopy was used to monitor the effect of PSMs produced in the intracellular space on the growth of the bacteria (GFP) and on the disruption of the neutrophil membranes (PI staining) over time. Again, the results indicated that PSMs contribute to the lysis of neutrophils after phagocytosis of *S. aureus*, as there was a clear delay in the timing of neutrophil lysis when neutrophils were infected with the *psm* triple mutant compared to the wild-type strain (**Figure 5**; MW2-GFP versus Δαβ*hld*-GFP). Additionally, we tested the

difference between neutrophil death and bacterial growth when neutrophils, after separate phagocytosis of wild type MW2 or Δαβ*hld*, were incubated in the same well. To enable separate visualization of both strains, a MW2Δαβ*hld* strain was used that constitutively expresses CFP (Δαβ*hld*-CFP; blue staining). When neutrophils were infected with MW2-GFP or Δαβ*hld*-CFP, killing of neutrophils infected with MW2-GFP was considerably advanced (**Figure 5**; MW2-GFP/Δαβ*hld*-CFP), as neutrophils containing green (MW2-GFP) bacteria stained PI-positive earlier then Δαβ*hld*-CFP-positive neutrophils. These data further confirm that PSMs contribute to lysis of the neutrophils after phagocytosis of *S. aureus*, and thereby promote staphylococcal escape from the neutrophil, as graphically depicted in **Figure 6.**

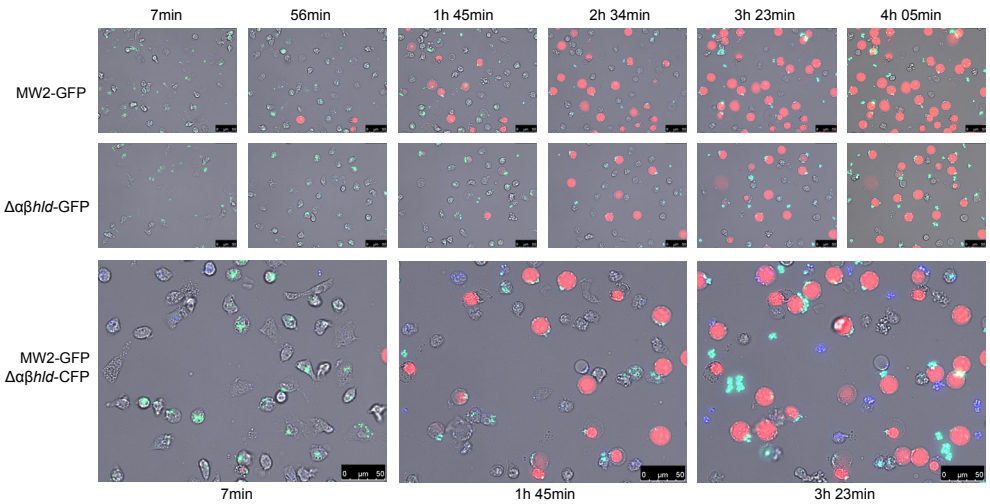

Figure 5. Time lapse analysis of neutrophil lysis and bacterial outgrowth of MW2 and the isogenic ΔαβHld mutant. Neutrophils were incubated with strain MW2-GFP, Δαβ*hld*-GFP or Δαβ*hld*-CFP, for 30 min, in the presence of 10% human serum to allow proper phagocytosis. After phagocytosis neutrophil killing was analyzed separately (upper two lanes), or together (bottom lane). A representative frame series out of three independent experiments is shown. Bright field (grey), GFP (green) CFP (blue) and PI (red) channels are combined.

Discussion

Neutrophils play a crucial role in clearing staphylococcal infections. Nevertheless, a fraction of *S. aureus* is capable of evading this innate immune defense, as it has evolved multiple and often redundant ways to evade neutrophils and remain undetected [19,20]. In addition, *S. aureus* makes use of a whole arsenal of cytolytic toxins. Leukocidins such as PVL [21], γ-hemolysins (HlgAB and HlgCB) [22], and the recently described leukocidin GH (LukGH) [23], also known as LukAB [24], are bi-component toxins that can specifically lyse neutrophils, most probably by formation of an octameric pore [25]. Of these bi-component toxins, PVL has recently received most attention, as this toxin is associated with CA-MRSA strains [26,27]. However, there has been controversy about the extent to which this toxin contributes to *S. aureus* pathogenesis [28,29]. Many of the controversial findings that

were obtained may be explained by species specificity, since murine leukocytes are highly resistant to cytolysis in vitro, whereas human and rabbit cells are highly susceptible to the toxin [30]. The species specificity of these bi-component toxins most likely is determined by the interaction with host G protein-coupled receptors, as was demonstrated recently for leukocidin ED (LukED) [31]. In contrast, *S. aureus* virulence attributed to PSMs is not species specific. Multiple cell types can be lysed, liposomes can be perforated [32] and genetic deletion of the PSMs impacts virulence in mice and rodents [8,14]. Together with the notion that PSMs are efficiently inhibited by the abundantly present lipoproteins, we hypothesized that PSMs could elicit their toxicity not as extracellular toxins but rather function from within the neutrophil after phagocytosis. Here, we show that PSMs are involved in neutrophil lysis after phagocytosis and that the PSMα operon is necessary and sufficient for this effect, inasmuch as *psm*α deletion strains have a significantly decreased capacity to lyse neutrophils after phagocytosis. Moreover, *psm*α deletion results in decreased bacterial survival and subsequent outgrowth, as compared to the wild type strain. In **Figure 6** we present a model of what happens to the *S. aureus* cell after it is phagocytosed by a neutrophil. By default phagocytosis will lead to destruction of the pathogen by the neutrophil. However, if *S. aureus* manages to produce enough PSMα in the phagosome, this will lead to lysis of the

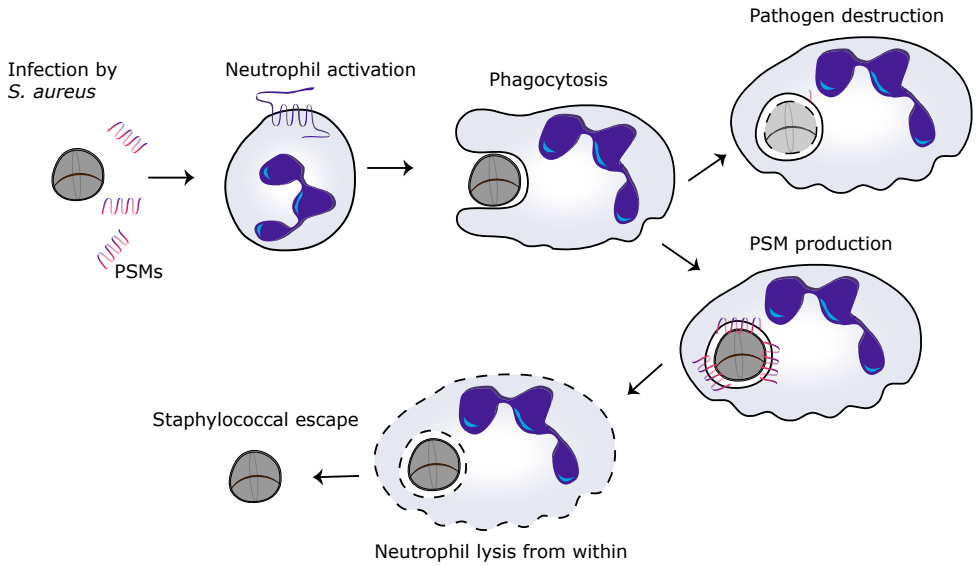

Figure 6. Staphylococcal PSMs contribute to phagosomal escape and intracellular lysis in the presence of serum. Graphic illustration depicting the role of staphylococcal PSMs during infection. Neutrophils recognize PSMs via their Formylated Peptide Receptor 2, are subsequently activated, attracted and will phagocytose the bacteria. Extracellular PSMs will be neutralized by serum lipoproteins. After phagocytosis, the pathogen can either be destroyed by the neutrophil or PSMα can contribute in the escape from the phagosome by lysing the neutrophil from the inside, which ultimately results in escape of *S. aureus* from the neutrophil.

neutrophil from the inside, resulting in survival and escape of the bacterium.

Geiger *et al*. recently showed that plasmid-based expression of the *psm*α or *psm*β operon results in increased escape from neutrophils after phagocytosis [18]. They also observed diminished lysis from the intracellular space using a *psm*α/*psm*β double mutant versus wild-type *S. aureus* Newman. These findings contradict ours, inasmuch as we did not detect a role of PSMβ peptides in that phenotype, which is in accordance with previous findings showing

no cytolytic activity of PSMβ peptides [8]. Notably, our experiments were performed with single isogenic *psm* deletion strains in a CA-MRSA strain, pinpointing lysis after phagocytosis to the PSMα peptides and allowing a conclusion on the contribution of the investigated toxins to their biological role in the background of an important clinical strain.

Multiple lines of evidence have accumulated in recent years demonstrating that *S. aureus* can survive within cells, or even use host cells as transport to disseminate from the site of infection [33,34,35]. One strategy involves the formation of small colony variants (SCVs), which is crucial for establishing chronic infections such as osteomyelitis [36]. Using this strategy, staphylococcal cells within epithelial cells are less susceptible to antibiotics and remain undetected by lowering their metabolism and growth and expression of virulence factors and regulators. However, to escape from the phagosome, staphylococci need to adopt a more virulent phenotype [37]. This switch is dependent on the intracellular activation of the *agr* system [38]. Inside host cells the concentration of the quorum sensing pheromone AIP can reach the critical concentration necessary for *agr* activation [39,40]. In line with these studies, we have shown with *psm*-GFP reporter constructs that the *psm*α operon is activated after phagocytosis of *S. aureus* by neutrophils [15]. Very recently, it was demonstrated that the stringent response, characterized by the rapid synthesis of (p) ppGpp as messenger of environmental stress conditions, is preceding the quorum sensing mechanism. Both systems appear to be crucial for up-regulating PSMs in the neutrophil phagosome and contribute to the lysis of neutrophils after phagocytosis and subsequent escape of the staphylococci [18].

The escape of *S. aureus* from endosomes in epithelial cells was reported to be dependent on β-toxin, β-PSM and Hld [41]. However, the corresponding studies were also only performed using plasmid-based artificial overexpression. Whether especially the PSMβ peptides contribute to that phenotype in a natural *S. aureus* background remains to be determined. Interestingly, while α-haemolysin (α-toxin, Hla) does not cause lysis of human neutrophils when added exogenously, a role for that toxin in phagosomal escape from human neutrophils and intracellular vacuoles of airway epithelial cells and was described previously [39]. In strain USA300 it was demonstrated that also LukGH plays a role in neutrophil lysis after phagocytosis [23]. Therefore, among different *S. aureus* strains multiple mechanisms, instead of a single virulence factor, may play a role in staphylococcal escape after phagocytosis; and different toxins may cause escape in different cell types.

Surprisingly, even in the total absence of PSMs (e.g. in *agr* or *psm*α*β*hld* mutants) strain MW2 can induce lysis and escape from neutrophil-mediated killing, although at lower levels. This is in accordance with previously published data indicating that staphylococci escape from neutrophils independently of toxins when assayed six hours post infection [7]. Importantly, next to its array of toxins, *S. aureus* appears to make use of the golden pigment staphyloxanthin and several catalases to survive the hostile environment of the neutrophil phagosome. Both these factors protect against reactive oxygen species by functioning as antioxidants [42]. Furthermore, modification of the cell wall by O-acetylation of peptidoglycan leads to resistance against lysozyme; and surface-located teichoic acids protect against antimicrobial peptides [43]. In *in vitro* model systems, *S. aureus* may use these factors to prolong survival until the neutrophil is weakened enough for the bacteria to escape. During infection of the host, the ratio of neutrophils to *S. aureus* is likely much more in favor of the neutrophil, making it necessary for *S. aureus* to employ aggressive methods

to survive the concerted attack of the innate immune system.

In conclusion, we show in this study that the PSMα peptides play an important role in the escape from neutrophils by killing the neutrophils from within. These data not only contribute to our understanding of the mechanisms virulent *S. aureus* strains use to circumvent killing by neutrophils, they also have major implications for the development of *S. aureus* vaccine strategies. First, strategies to specifically target PSM peptides will have to consider that these virulence factors exert a major function in pathogenesis within the neutrophil, which might make strategies employing anti-PSM antibodies difficult. Second, our findings may explain why traditional active vaccination strategies against *S. aureus* have so far failed, inasmuch as efficient killing of the neutrophil after phagocytosis undermines the increase of opsonophagocytosis by neutrophils, by which those vaccines mainly are supposed to function [44].

Acknowledgments

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Supporting information

Phagocytosis: serum

No phagocytosis: buffer

No Phagocytosis: CytD

No Phagocytosis: HI serum Flipr-L

QR codes of supplementary movies. Time lapse movies of neutrophil lysis and bacterial outgrowth. Analysis of neutrophil phagocytosis of CA-MRSA strain MW2 by time lapse fluorescence microscopy. Neutrophils were incubated with strain MW2, which constitutively expresses GFP (MW2-GFP) for 30 min, in the presence of human serum (phagocytosis), or absence of opsonin (Buffer) (No phagocytosis), or serum with addition of cytochalasin D (CytD), or heat-inactivated serum together with pre-incubation of neutrophils with FLIPR-Like (FcγR-inhibitor) (HI-HPS+Flipr-L). A representative frame series out of three independent experiments is shown. Brightfield (grey), GFP (green) and PI (red) channels are combined.

6

Pneumococccal Immune Evasion: ZmpC inhibits Neutrophil Influx

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Abstract

Neutrophil recruitment is essential in clearing pneumococcal infections. The first step in neutrophil extravasation involves the interaction between P-selectin on activated endothelium and P-Selectin Glycoprotein 1 (PSGL-1) on neutrophils. Here, we identify pneumococcal Zinc metalloproteinase C as a potent inhibitor of PSGL-1. ZmpC degrades the N-terminal domain of PSGL-1, thereby disrupting the initial rolling of neutrophils on activated human umbilical vein endothelial cells. Furthermore, mice infected with wild-type strain in the model of pneumococcal pneumonia showed lower lungs neutrophil infiltration compare to animals infected with ZmpC mutant. In addition, we confirmed the association of *zmpC* with serotype 8 and 11A and found an association with serotype 33F as well. In conclusion, we report PSGL-1 as a novel target for ZmpC and show that ZmpC inhibits neutrophil extravasation during pneumococcal pneumonia.

Introduction

Streptococcus pneumoniae (pneumococcus) is the most common bacterial respiratory pathogen. Although carriage of *S. pneumoniae* in the naso-oropharynx is usually asymptomatic, the bacterium can spread from this location and cause a range of diseases varying from mild otitis media to potentially fatal pneumonia [1]. Each year, over 1.6 million people die from invasive pneumococcal disease (IPD) that is manifested by sepsis or meningitis [2]. Control of *S. pneumoniae* infection involves recognition of the pathogen, leading to recruitment of phagocytic cells and clearance of the bacterium [3]. To counteract this response *S. pneumoniae* relies on virulence factors that influence the uptake and phagocytic killing by those recruited phagocytes [4].

Another mechanism of immune evasion is interfering with the recruitment of leukocytes to the site of infection [5]. Leukocyte recruitment is a multi-step process initiated by recognition of the invading pathogen via pattern recognition receptors. Secreted cytokines and chemokines activate the endothelium lining blood vessels, inducing up-regulation of selectins, essential in initiation of neutrophil recruitment. P-selectin stored in Weibel-Palade bodies within resting endothelial cells, and E-selectin synthesized *de novo*, is then translocated to the apical cell membrane, where they transiently bind to PSGL-1 of the leukocytes [6,7]. P-selectin is the major ligand for PSGL-1, however also E-selectin is known to interact with PSGL-1. Post-translational modifications such as *O*-glycosylation and tyrosine sulfation in the N-terminal region of PSGL-1 contribute to the efficient binding to selectins [8,9,10]. PSGL-1 is expressed on most leukocytes and mediate neutrophil tethering and rolling via transient interactions with selectins in combination with shear stress by the blood flow [11]. These initial steps are followed by activation of the neutrophil resulting in conformational changes in β2-integrins and neutrophil arrest [12]. Consequently, it leads to neutrophil transmigration through the endothelial cell layer, chemotaxis towards the site of infection and clearance of invading pathogens [12].

S. pneumoniae secretes up to four zinc metalloproteases (Zmp) depending on the strain [13]. The first discovered Zmp is IgA protease (ZmpA) that specifically cleaves human IgA1 in the hinge region, thereby preventing antibody-mediated immunity [14,15] while enhancing pneumococcal adhesion to host mucosal cells [16]. Several studies have identified IgA protease as important virulence factor in *in vivo* models for sepsis and pneumonia [17,18]. Although the specific targets of ZmpB and ZmpD remain to be determined, a role in pneumococcal virulence has been shown for ZmpB as mutants devoid of *zmpB* were attenuated in murine models of infection [19]. Moreover, a ZmpB-based vaccine protects against pneumococcal diseases in mice [20]. ZmpC has the capacity to cleave matrix metalloproteinase-9 (MMP-9) [21] and to stimulate shedding of syndecan-1 [22] and MUC16 [23]. In addition, two studies using *zmpC*-knockout strains have identified a role of ZmpC in pneumococcal virulence [18,21]. IgA protease and ZmpB were present in all pneumococcal isolates examined so far, whereas ZmpC and ZmpD were detected in fractions of *S. pneumoniae* strains tested [13,24].

This study was conducted to identify novel virulence factors of *Streptococcus pneumoniae.* Here, we identify ZmpC as a potent PSGL-1 inhibitor *in vitro* and demonstrate its activity *in vivo.*

Materials and Methods

Ethic statement
Human leukocytes were isolated after written informed consent was obtained from all subjects in accordance with the Declaration of Helsinki. Approval was obtained from the medical ethics committee of the UMC Utrecht. The Utrecht University Ethical Committee for Animal Experimentation approved the animal study protocols, in accordance with the advice of the Central Committee on Animal Experimentation (Approval 2011.II.05.078) and the Dutch Law on Animal Experimentation (art. 18a).

Bacterial Strains and Culture Conditions
Streptococcus pneumoniae strains (**Table S1**) were grown overnight on 5% sheep blood agar plates (Oxoid). For secretome production, strains were cultured till OD_{660} 0.5 in IMDM without phenol red, supplemented with 10% Todd-Hewitt broth. Culture supernatants were clarified by centrifugation and filter-sterilized through 0.2 µM filters (Millipore). Supernatants were concentrated with 10 kD or 100 kD cut-off amicon ultracentrifugation units (Millipore). Protease mutant strains devoid of the three proteinases were generated in the TIGR4 (supplementary methods and **Table S1-2**). For animal experiments, strains were grown in Brain-Heart-Infusion broth and culture aliquots supplemented with 10% glycerol were stored at -70^{o}C. Prior to animal experiments, stocks were thawed, washed and resuspended in saline at the appropriate CFU/mL.

Antibody competition assay
Polymorphonucleated cells (PMN, neutrophils) and peripheral blood mononucleated cells (PBMC, monocytes and lymphocytes) from healthy volunteers were isolated as described [26] and mouse neutrophils were isolated from the bone marrow of 3-month-old female C57Bl/6 mice (Harlan) as described [53]. HL-60 cells were cultured and treated with 10 or 50 mM sodium chlorate as described [25]. Cells were incubated with pneumococcal culture supernatants or purified ZmpC diluted in RPMI (Invitrogen) supplemented with 0.05% human serum albumin (HSA; Sanquin) for 30 min at 37°C or on ice. Cells were washed with RPMI-HSA and tested for binding of different monoclonal antibodies (mAb) using a FACS Calibur (BD biosciences) as described [26,53]. Where indicated, cells were incubated prior to ZmpC treatment with 0.2 U/mL sialidase (Roche) for 45 min at 37°C. Alternatively, cells were incubated with ZmpC in 10% heat-inactivated (20 min 56ºC) serum. In separate experiments, neutrophils were incubated with increasing concentrations of ZmpC or anti-PSGL-1 mAbs PL1 (Bioconnect) or KPL1 (BD) on ice for 30 min, washed and stained with 1 µg/mL P-selectin/Fc chimera (R&D Systems) for 30 min on ice. Bound P-selectin/Fc was detected with 5 µg/mL goat anti-human IgG-FITC (Bio-rad).

Confocal analysis
Neutrophils (2.5×10^5) were incubated with buffer or 1 µg/mL ZmpC for 30 min at 37°C in RPMI/0.05% HSA. Cells were washed and stained with KPL1 or PL2. Bound antibodies were detected using FITC-labeled goat anti-mouse (Dako). After washing, neutrophils were stained with a DiD membrane dye (Invitrogen). Cells were analyzed using a Leica TSC SP5 confocal microscope as described.[53]

Cleavage of PSGL-1
Recombinant PSGL-1/Fc 10 µg/mL was incubated with purified ZmpC or concentrated TIGR4 supernatant in PBS for 15 min at 37°C. Samples were separated by SDS-PAGE, transferred to a PVDF membrane, and blocked with 4% skimmed milk in PBS + 0.05% Tween. Next, blots were incubated with PSGL-1 antibodies PL2 or KPL1 (0.5 µg/mL) for 1 h at 37°C. As secondary antibody a HRP-labeled goat anti-mouse IgG (Biorad) was used. Bands were visualized by enhanced chemiluminescence (Amersham).

Static adhesion assay
Static adhesion of neutrophils was performed, as described [26], with minor modifications. Calcein-labeled cells (3×10^5/mL) were incubated with ZmpC for 15 min on ice in HBSS with 0.05% HSA, before addition to recombinant P-selectin coated plate for 15 min at RT. After washing, adhered cells were quantified using a platereader fluorometer (FlexStation; Molecular Devices) at 450 nm excitation and 530 nm emission.

Neutrophil rolling
Rolling experiments with neutrophils were performed, as described [26]. Neutrophils were pretreated with 3 or 1 µg/mL ZmpC for 10 min at 37°C. Human umbilical vein endothelial cells (HUVECs) were stimulated with 100 µM histamine (Sigma) for 3 min to induce expression of P-selectin, and neutrophils were immediately perfused at 0.8 dyn/cm2. Number of adherent cells was visualized using a camera, and recorded images were analyzed using the program Optimas 6.1 (Media Cybergenetics Systems).

Mice infection model
Six- to eight-week-old Female C57BL/6 (Harlan) mice were acclimatized for one week prior to use. Mice were lightly anaesthetized with 5% Isoflurane and a single dose of 35 µl PBS containing 5×10^6 CFU *S. pneumoniae* TIGR4S or TIGR4*ΔZmpC* was administered into the nostrils. The inoculum dose was confirmed by viable count following infection. At 8 h after infection, mice were sacrificed, blood was obtained via cardiac puncture, BAL fluids were collected by flushing the lungs twice with 600 µL sterile PBS. The lungs were removed and subsequently homogenized in 1 mL PBS. From both BAL and lung homogenates CFU bacterial counts were determined by viable count on blood agar plates supplemented with gentamicin at 5 mg/L (Oxoid). Total BAL leukocyte number was determined with coulter counter (Beckman). The number of infiltrating neutrophils was enumerated from the percentage BAL positive GR-1^+ F4/80^-cells measured by flow cytometry, as described.[54] Additional staining on BAL cells was performed to measure the presence of PSGL-1 with Rat anti-mouse PSGL-1 (clone 4 RA10) antibody.

MLST analysis
Multi Locus Sequence Typing (MLST) was performed as described by Enright and Spratt. The assignment of sequence types was carried out using online software at the pneumococcal web page: (www.spneumoniae.mlst.net).

Statistical analysis
Statistics were analyzed with a two-tailed Student's t-test. Values below $P<0.05$ were considered significant.

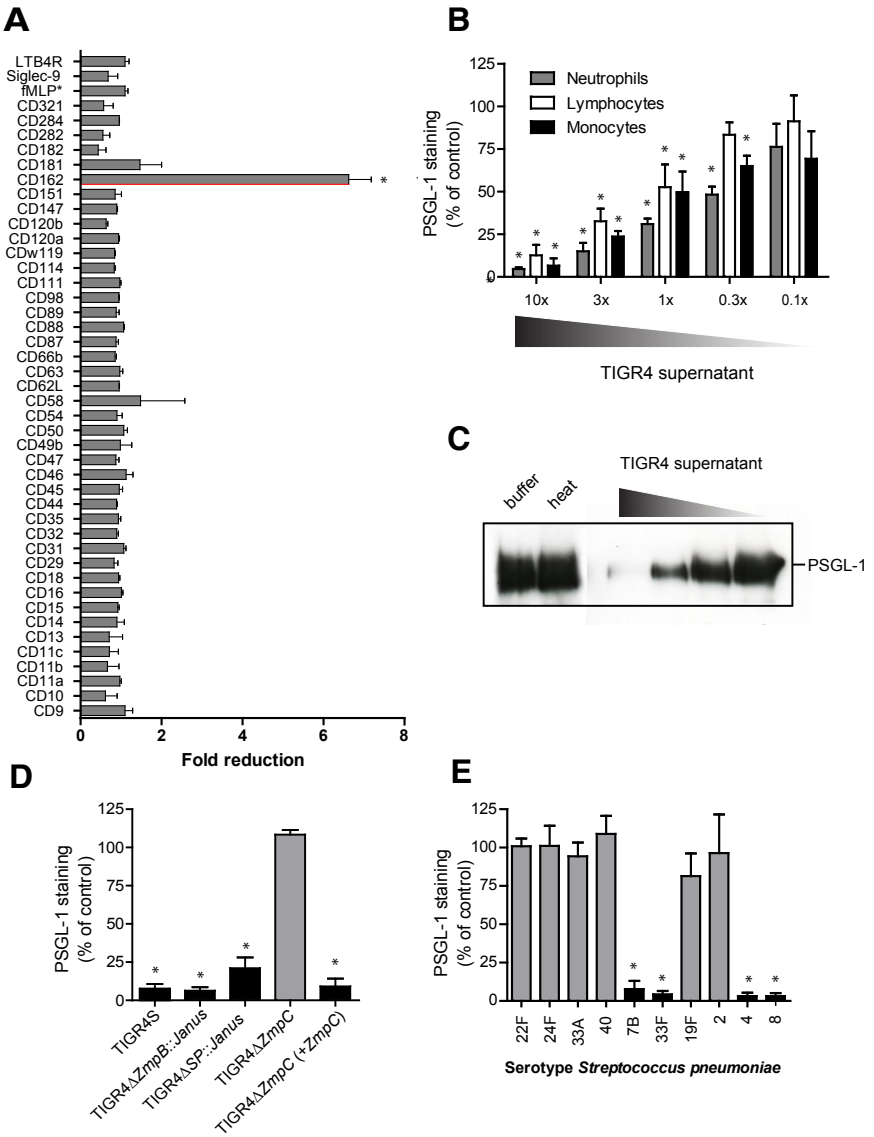

Figure 1. ZmpC prevents mAb binding to PSGL-1. (A) mAb competition assay. Neutrophils were incubated with 30× concentrated *Streptococcus pneumoniae* TIGR4 culture supernatant or buffer for 30 min at 4°C. After washing, cells were stained with a panel of blocking antibodies against surface-expressed receptors. The fold reduction of mAb binding was calculated by dividing the mean fluorescence of buffer-treated cells by TIGR4 supernatant treated cells. (B) TIGR4 culture supernatant blocks mAb binding in a dose-dependent manner. Leukocytes were incubated with various concentrations of TIGR4 culture supernatant, ranging from 10× to 0.1× concentrated, for 30 min at 4ºC. The percentage PSGL-1 staining was determined by comparison of mean fluorescence with untreated cells. (C) Western analysis of recombinant human PSGL-1/Fc incubated for 15 min at 37ºC with 0, 10× concentrated heat-inactivated or 10, 3, 1 and 0.3× concentrated TIGR4 culture supernatant. PSGL-1/Fc was detected with the anti-PSGL-1 mAb KPL-1. PSGL-1 mAb inhibition assay of (D) 10× concentrated culture supernatants of isogenic TIGR4 mutants or (E) various *S. pneumoniae* clinical isolates with different capsular serotypes. (D and E) Black bars represent strains were *zmpC* is present, confirmed by PCR. All figures represent the mean ± s.e.m. of three separate experiments using different donors. Background was determined using an isotype control. (ABDE) * indicates statistical significant difference of P<0.01, for supernatant treated cells compared to buffer treated cells

Results

Streptococcus pneumoniae supernatant can cleave PSGL-1

To find novel inhibitors of innate immunity in *S. pneumoniae*, a multi-screening assay for surface-expressed receptors on neutrophils was performed. All mAbs in this screen target receptors that have distinct functions in innate immunity, for instance; in pattern recognition, activation, transmigration, chemotaxis and phagocytosis. For this purpose, the mid-log culture supernatant of invasive *S. pneumoniae* strain TIGR4 (Tettelin *et al*, 2001) was incubated with freshly isolated neutrophils for 30 min at 4°C and then binding of several mAbs was examined. Marked inhibition of the antibody directed against PSGL-1 (CD162) was observed, whereas the mAb-binding to all other 44 receptors tested remained unaffected (**Figure 1A**). Various dilutions of the TIGR4 supernatant showed a dose-dependent inhibition of the PSGL-1 antibody binding not only to neutrophils, but also to monocytes and lymphocytes (**Figure 1B**). Subsequent degradation of recombinant PSGL-1/Fc incubated with the TIGR4 supernatant indicated the involvement of a protease in cleaving PSGL-1. This degradation was not observed when the supernatant was heat-inactivated (**Figure 1C**). Fractionation of the supernatant by size-exclusion chromatography resulted in several fractions that inhibited PSGL-1 antibody binding (**Figure S1A,B**). Examination of the active fractions by SDS-PAGE revealed multiple protein bands at approximately 200 kDa with corresponding activity (**Figure S1A,B**). The proteins of interest were identified by mass-spectrometry as; ZmpB, ZmpC and Serine protease of *S. pneumoniae*. Genetic deletion strains of these proteases were generated in the TIGR4 and concentrated supernatants of mutant cultures were examined for inhibition of anti-PSGL-1 binding to neutrophils. Supernatants from TIGR4 lacking the *zmpB* or Serine protease encoding gene still showed inhibition of PSGL-1 antibody binding. In contrast, deletion of *zmpC* completely abrogated the inhibition of PSGL-1 antibody binding (**Figure 1D**). Genetic complementation of *zmpC* restored the wild type phenotype, indicating ZmpC was the likely candidate with anti-PSGL-1 activity.

Ten clinical pneumococcal isolates of different capsule types were screened for the presence of *zmpC* and their ability to compete with anti-PSGL-1. The ability of the culture supernatants of these strains to block PSGL-1 antibody binding to neutrophils completely correlated with the presence of *zmpC*, detected by PCR (**Figure 1E**). Taken together, these data clearly show that some *S. pneumoniae* strains prevent binding of an antibody directed against PSGL-1, and ZmpC is the protease responsible for the effect.

Pneumococcal ZmpC cleaves PSGL-1

To further address the interaction between ZmpC and PSGL-1, we adopted a biochemical approach to purify sufficient amounts of pure protein. Similar to Chen [22] we used the TIGR4ΔZmpB mutant strain to purify ZmpC. Starting with 4L of culture supernatant, over 1 mg 95% pure ZmpC was purified by chromatography (**Figure S1C**). Freshly-isolated human neutrophils, monocytes and lymphocytes were treated with ZmpC, and subsequently three PSGL-1 antibodies recognizing different epitopes were used to pinpoint the ZmpC cleavage site on PSGL-1. Anti-PSGL-1 antibodies KPL1 and PL1 recognize an N-terminal epitope on PSGL-1, whereas PL2 recognizes a membrane-proximal epitope [9] (**Figure 2A**). Analysis of

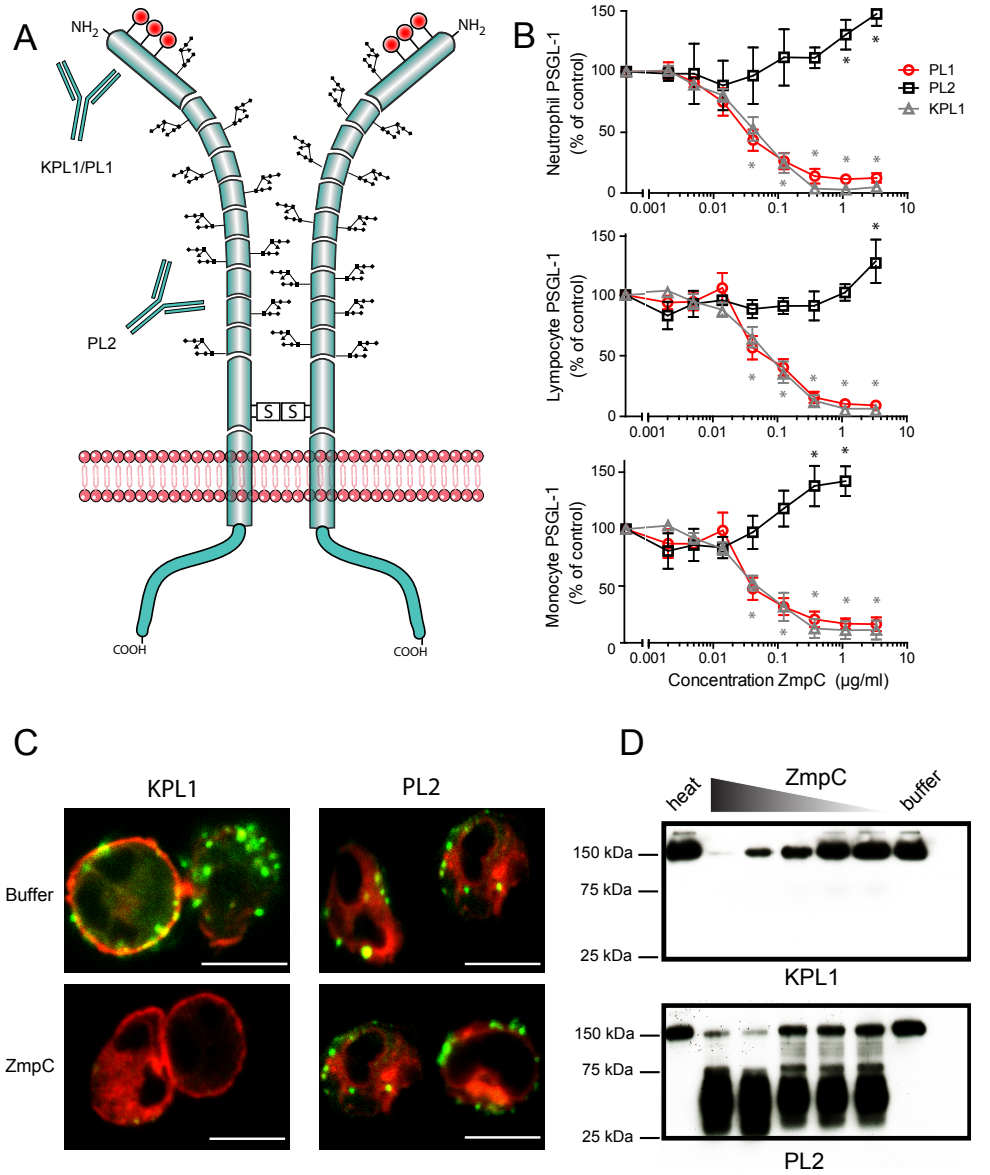

Figure 2. PSGL-1 cleavage by ZmpC. (A) Schematic representation of dimeric PSGL-1 as expressed on cells. Indicated are the binding epitopes of KPL1 (aa 42-58), PL1 (aa 49-62) and PL2 (188 to 235).[42,43] (B) Human neutrophils, monocytes and lymphocytes were treated with increasing concentrations of ZmpC for 30 min at 4°C before staining with the PSGL-1 antibodies PL1, PL2 and KPL1 and subsequent incubation with FITC-labeled goat anti-mouse IgG. The PSGL-1 staining of untreated cells was set to 100%. Data represent the mean ± s.e.m. of three independent experiments. For PL1 and KPL1, * indicate statistical significant difference of $P<0.05$, for PL2 in the opposite direction, all versus buffer treated cells. (C) Confocal images of human neutrophils stained with antibodies KPL1 and PL2 (green). Cells were treated with 3 µg/mL ZmpC and PSGL-1 was stained as described above. Membranes were stained with DiD (red). (D) Western analysis of recombinant human PSGL-1/Fc incubated for 15 min at 37ºC with 3 µg/mL heat inactivated or 3, 1, 0.3, 0.1, 0.03, and 0 µg/mL ZmpC. PSGL-1 was detected with KPL1 or with PL2 antibodies. Shown is one representative of 3 independent experiments. * indicate statistical significant difference of $P<0.01$.

PSGL-1 staining on leukocytes revealed that ZmpC cleaved off the N-terminal epitope of PSGL-1, as diminished KPL1 and PL1 antibody binding was observed (**Figure 2B**). An IC_{50} of 50 ng/mL was calculated from the inhibition of KPL1 or PL1 binding to neutrophils. In contrast, the PL2 epitope on PSGL-1 remained available for antibody binding. This indicates that the ZmpC cleavage site is located between the N-terminal KPL1/PL1 epitope and the membrane-proximal PL2 epitope on PSGL-1. Corresponding results were obtained by confocal microscopy (**Figure 2C**); ZmpC cleaved-off the KPL1 epitope while it did not affect the PL2 epitope.

We further investigated whether serum proteins influence the ZmpC cleavage of PSGL-1. Pre-incubation of ZmpC with 10% human serum did not result in a decreased cleavage of PSGL-1 (**Figure S1D**). In addition, we investigated the cleavage of recombinant PSGL-1/Fc by Western analysis. ZmpC removes the N-terminus of PSGL-1, as the reactivity of KPL1 with PSGL-1/Fc was lost while PL2 still recognized multiple degradation products between 150 kDa and 25 kDa (**Figure 2D**). Thus, ZmpC removes the part of PSGL-1 essential for ligand interaction with P-selectin.

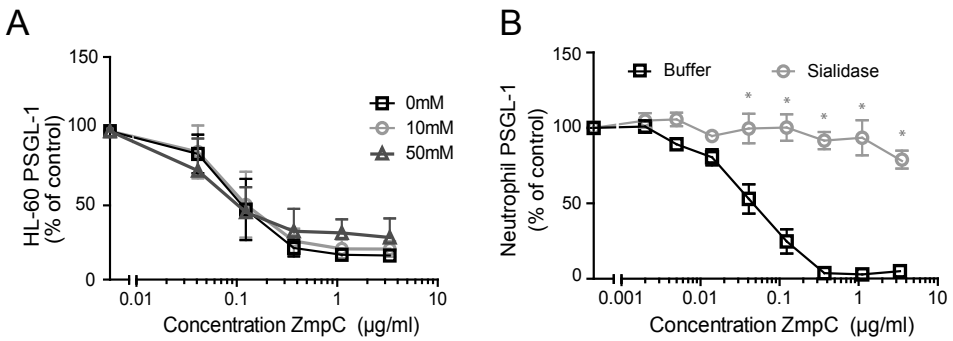

Figure 3. Influence of post-translational modifications of PSGL-1 on the cleavage by ZmpC. (A) The effect of pre-treatment of HL-60 cells with sodium chlorate on ZmpC inhibition of PSGL-1 staining on HL-60 cells. (B) The effect of sialidase treatment for 45 min at 37ºC on ZmpC-induced cleavage of PSGL-1 on human neutrophils. PSGL-1 on human neutrophils or HL-60 cells was stained with PE-conjugated mAb KPL1 and measured by flow cytometry. The percentage PSGL-1 staining was determined by comparison of mean fluorescence with untreated cells. Data represent the mean ±s.e.m. of three independent. * indicates statistical significant difference of P<0.01

ZmpC cleavage of PSGL-1 is dependent on proper O-linked glycosylation

The N-terminal region of PSGL-1 is critical for PSGL-1 binding to P- and E-selectins. Post-translational modifications such as sialyl Lewis x-containing *O*-glycosylation and tyrosine sulfation in the N-terminal region of PSGL-1 determine the efficiency of binding to selectins. Therefore, we investigated whether post-translational modifications of PSGL-1 influence its cleavage by ZmpC. First, we analyzed the requirement of sulfation for the cleavage of PSGL-1. PSGL-1-expressing HL-60 cells were treated with sodium chlorate, blocking the sulfation of tyrosines, without influencing the surface expression of PSGL-1. [25] Sodium chlorate treatment did not affect the ZmpC-induced inhibition of PSGL-1 antibody binding (**Figure 3A**). Next, neutrophils were treated with sialidase before ZmpC incubation. Sialidase treatment removes sialic acid, a component of sialyl Lewis x, from PSGL-1 and thereby abrogates the binding of PSGL-1 to its natural ligand P-selectin [26]. ZmpC was unable to cleave PSGL-1 on

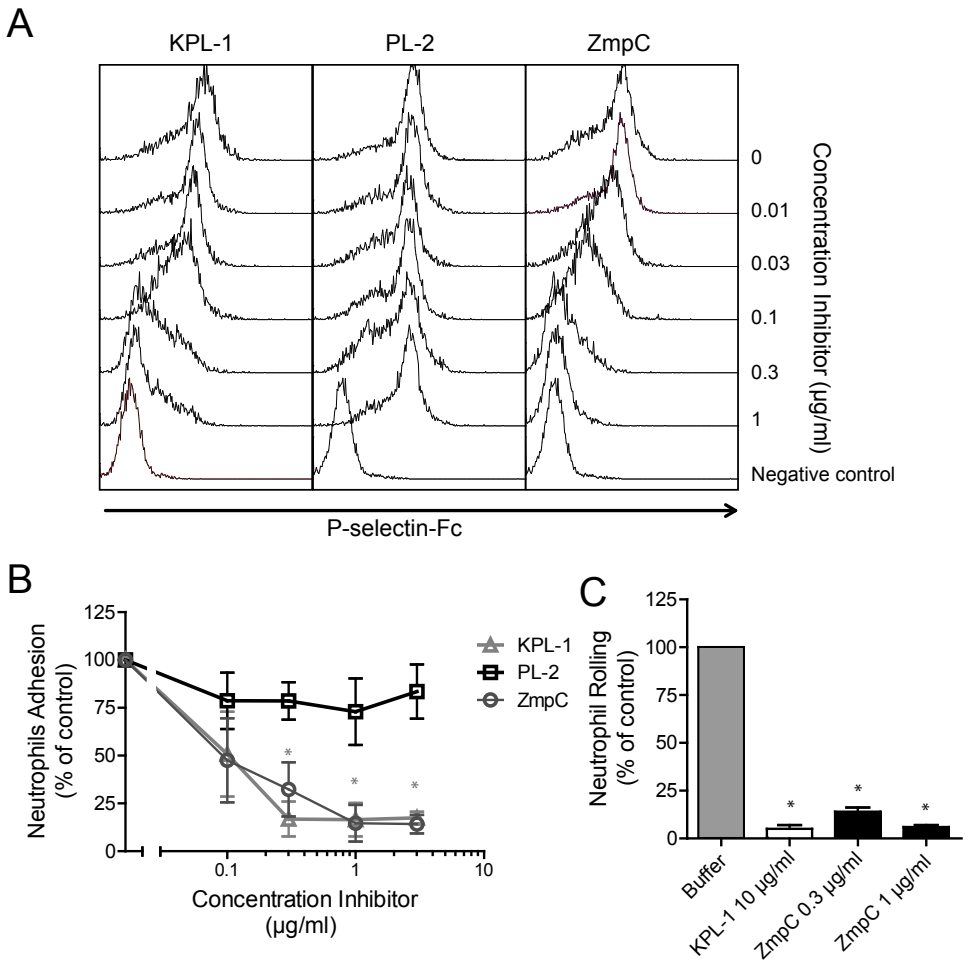

Figure 4. Functional inhibition by ZmpC. (A) Flow cytometric analysis of P-selectin binding to human neutrophils in the presence of functional inhibitors KPL1 (blocking mAb), PL2 (non-blocking mAb) and ZmpC. P-selectin/Fc was incubated for 30 min together with the inhibitors on ice. P-selectin/Fc was detected with goat anti-human IgG-FITC. Shown is one representative of 3 independent experiments. (B) The effect of ZmpC on adhesion of human neutrophils under static conditions. Calcein-labeled human neutrophils were allowed to adhere for 20 min at RT on P-selectin coated plates. Cells were pretreated with ZmpC, KPL1 (blocking mAb), or PL2 (non-blocking mAb). Adherence to P-selectin was determined by measuring the fluorescence after several washes with PBS. Data represent the mean ±s.e.m. of three independent experiments. (C) The effect of ZmpC on rolling adhesion of human neutrophils on freshly isolated human umbilical vein endothelial cells (HUVECs). HUVECs were cultured on glass coverslips. After stimulation of HUVECs with 100 µM histamine for 3 minutes, neutrophils treated with 10 µg/mL KPL1 (blocking mAb) or 1 to 3 µg/mL ZmpC were perfused at 0.8 dyn/cm^2 for 5 minutes. After washing for 1 minute, accumulated neutrophils were quantified. The data represent relative accumulation of neutrophils compared with control-treated cells and are the mean ± s.e.m. of at least 3 independent experiments. * indicated statistical significant difference of P<0,05, versus controls; (B) PL2 or (C) buffer treated cells.

neutrophils after sialidase treatment (**Figure 3B**). Thus, for cleavage by ZmpC, PSGL-1 needs to be properly O-glycosylated, whereas PSGL-1 sulfation is not necessary.

Functional inhibition by ZmpC

Binding of PSGL-1 to P-selectin plays an important role in neutrophil migration towards a site of infection. Therefore, we investigated whether this binding was affected by ZmpC cleavage. ZmpC interfered with the binding of P-selectin/Fc to PSGL-1 on neutrophils as potent as the blocking antibody KPL1 (**Figure 4A**). As a control, the non-blocking antibody PL2 did not show inhibition of P-selectin/Fc binding. Accordingly, ZmpC inhibited the adhesion of neutrophils under static conditions to P-selectin as potently as KPL1, whereas PL2 did not have an effect (**Figure 4B**). In the bloodstream, shear stress and the intimate interaction of neutrophil PSGL-1 with endothelial expressed P-selectin mediate the rolling phenomenon.

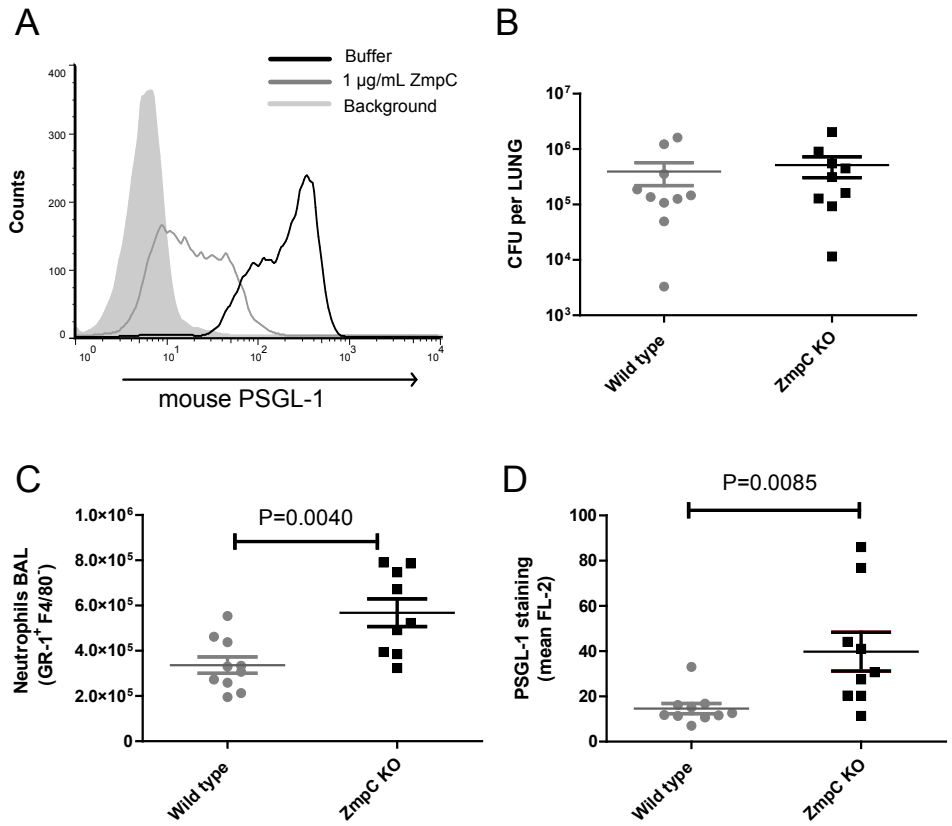

Figure 5. ZmpC functions as inhibitor of neutrophil accumulation *in vivo.* (A) Histogram of mouse bone marrow derived neutrophils treated with various concentrations ZmpC. PSGL-1 levels were detected with rat anti-mouse mAb (4RA10) and measured by flow cytometry. Data represent one representative of 3 different experiments. Ten C57BL/6 mice were inoculated intranasally with 5×10^6 CFU TIGR4S or the isogenic mutant TIGR4Δ*ZmpC*. (B) Bacterial loads 8 hours after onset of disease. Total number of CFUs per lungs was determined by plating lung homogenates and bronchial alveolar lavage fluids (BAL). (C) The number of neutrophils infiltrating the lungs of infected mice. Data represent the total cell number of the BAL × the percentage of neutrophils obtained by staining for Gr-1 and measured by flow cytometry. (D) Total BAL fluids of mice infected with wild type TIGR4S or mutant TIGR4Δ*zmpC* were stained for PSGL-1 as described above. P-values were obtained by a two-tailed Student's t-test.

Table 1 Distribution of *zmpC* gene among *Streptococcus pneumoniae* strains isolated from invasive pneumococcal disease in the Netherlands between 2004 and 2009.

	ZmpC		
Serotype	**Absent**	**Present**	**Total**
1	6		6
3	7		7
4	2	2	4
6A	6		6
6B	11		11
7B	1		1
7C	1		1
7F	20		20
8		12	12
9N	4		4
9V	24		24
10A	3		3
10F	1		1
11A		13	13
11F	1		1
12F	2		2
14	23		23
15B	4		4
15C	1		1
16F	1		1
17F	1		1
18A	1		1
18B	1		1
18C	6		6
19A	4		4
19F	2		2
22F	3		3
23A	2		2
23F	10		10
24F	1		1
27	1		1
28A	1		1
29	1		1
31	1		1
33A	1		1
33F		11	11
34	1		1
35B	2		2
37	1		1
38	2		2
Total	**160**	**38**	**198**

We simulated this interaction *in vitro*, by perfusing neutrophils over histamine-activated HUVECs in presence or absence of ZmpC or KPL1. After 5 minutes of treatment, the number of rolling neutrophils was monitored. Pre-incubation with KPL1 strongly affected the number of rolling neutrophils on HUVECs (**Figure 4C**). ZmpC also blocked the rolling interaction for over 80% at 0.3 µg/mL (**Figure 4C**). Thus, ZmpC potently interferes with the binding of PSGL-1 to P-selectin, both under static and flow conditions mimicking the *in vivo* situation.

***In vivo* model of neutrophil infiltration**

Multiple studies have shown that ZmpC is an important virulence determinant *in vivo.* [18,19,21] Our *in vitro* data suggest a clear role for ZmpC in the pathogenesis of *S. pneumoniae* by cleaving PSGL-1 and thereby hampering recruitment of neutrophils. To test whether cleavage of PSGL-1 is indeed an important *in vivo* virulence mechanism, we started with confirming that also mouse PSGL-1 is cleaved by ZmpC (**Figure 5A**). Then, the effect of ZmpC was examined in a pneumonia model in C57Bl/6 mice through infection with wild type *S. pneumoniae* TIGR4 or its isogenic *zmpC* mutant. As demonstrated before, acute pneumococcal pneumonia is accompanied by massive neutrophil influx 8 hours after disease onset. [27,28,29] Here, the intranasal inoculation of mice with wild type TIGR4 resulted in a significantly lower amount of neutrophils recruited to lung lumen compared to the isogenic *zmpC* mutant (**Figure 5C**), whereas no difference in neutrophil recruitment could be observed in the lung interstitial tissue (**Figure S1F**). No bacteremia was observed in the blood obtained after cardiac puncture. Importantly, at this early onset of disease, no difference in total bacterial load was observed (**Figure 5B**). Furthermore, BAL cells isolated from mice infected with wild type TIGR4 showed lower staining for PSGL-1 compared to BAL cells from mice infected with the *zmpC* mutant, implying *in vivo* proteolysis of PSGL-1 by ZmpC (**Figure 5D**). In conclusion, ZmpC impairs neutrophil infiltration *in vivo* most likely through proteolytic degradation of PSGL-1.

Epidemiology

Previously, presence of the *zmpC* has been linked to pneumococcal strains of serotypes 8 and 11A.[13] We assessed by PCR the presence of *zmpC* in a collection of 198 strains isolated from IPD in the Netherlands between 2004 and 2009. We confirmed the association of *zmpC* with serotype 8 and 11A and found it to be associated with serotype 33F as well (Table 1). Multi Locus Sequence Typing (MLST) identified serotype 33F strains tested in this study as members of clonal complexes (CC) CC100 or CC1012. (www.spneumoniae.mlst.net) In addition, we measured the presence of antibodies against ZmpC in healthy donors. We detected anti-ZmpC IgG in serum samples from all 12 individuals tested (**Figure S1E**). It indicates that the exposure to antigen is common despite *zmpC* being present in minority of *S. pneumoniae* strains circulating in human population.

Discussion

Neutrophils play a crucial role in the innate immune system, since they are important for rapid clearance of bacteria. During infection, local activation of the complement system and

production of chemokines by neighboring cells lead to the recruitment of neutrophils to the site of infection. Several secreted bacterial proteins have been shown to target the very first step of neutrophil extravasation; the P-selectin - PSGL-1 interaction. Staphylococcal superantigen-like 5 (SSL5) binds and inhibits PSGL-1 mediated rolling on HUVECs [26]. ImpA of *Pseudomonas aeruginosa* and serine protease autotransporters of Enterobacteriaceae (SPATEs) were shown to cleave multiple glycoproteins including PSLG-1 *in vitro*, thereby expected to contribute to bacterial pathogenesis [30,31]. Here, we show that ZmpC of *S. pneumoniae* specifically cleaves PSGL-1 and thereby inhibits neutrophil rolling on HUVECs. Neutrophil attraction is crucial in controlling pneumococcal infection [32]. Hampering the initial interaction in leukocyte recruitment would therefore lead to enhanced virulence. The importance of ZmpC as a virulence factor has clearly been demonstrated *in vivo* in previous studies [18,19,21]. These studies have indicated a role for ZmpC in activation of MMP9 and this mechanism has been correlated to pneumococcal disease severity and progression, through proteolytic tissue remodeling [21]. However, the presence of ZmpC homologs in other streptococcal species did not correlate with the cleavage of MMP9, suggesting that there are other substrates for this enzyme [24]. In the current study, we demonstrate that ZmpC efficiently cleaves PSGL-1 *in vitro* and *in vivo,* providing an additional mechanism by which ZmpC contributes to virulence of *S. pneumoniae*.

Of all proteins contributing to *S. pneumoniae* virulence, ZmpC is one of the least prevalent among *S. pneumoniae* strains [33]. Although sporadically detected in pneumococcal isolates of various capsular types, *zmpC* is reported at particular high frequency (>90%) among *S. pneumoniae* isolates of serotypes 8 and 11A [13,23]. Our screening of pneumococcal isolates collected between 2004 and 2009 from patients with IPD in the Netherlands confirmed strong association between *zmpC* and these two serotypes, and we additionally found that all tested serotype 33F strains carry this gene. The majority of serotype 8 and 11A isolates form a single cluster of clonally related isolates. The cluster is composed of two CCs dominated either by serotype 8 (CC53) or 11A (CC62) strains (Camilli *et al*., 2006) (www.spneumoniae.mlst.net). Serotype 33F isolates of CC1012, which we found positive for *zmpC* were double-locci variants of sequence type ST1180 belonging to CC62. The 33F isolates are genotypically related to the major CC53/CC62 clonal cluster of serotype 8 and 11A strains. Interestingly, although CC1012 is dominated by strains of serotype 33F, it also contains serotype 8 and 11A strains, and a similar pattern of serotype distribution is observed for clonally distant CC100 (www.spneumoniae.mlst.net). In addition, analysis by Deltarho-web shows a highly dissimilar DNA dinucleotide frequency signature of *zmpC* compared to the rest of the TIGR4 genome. The GC content of *zmpC* is lower than that of the mean of the TIGR4 genome (35.7% vs 39.7%) with only 12% of the fragments of similar size of the *S. pneumoniae* genome having a lower GC content [34]. Together, these features favor the recently suggested hypothesis of recent introduction of *zmpC* into *S. pneumoniae* and clonal spread of the gene [24,33].

Epidemiological surveillance studies investigating the impact of heptavalent conjugated polysaccharide pneumococcal vaccine (PCV-7) identified serotype 8, 11A and 33F among capsular types frequently carried by immunized children [35,36]. Although serotype-associated invasiveness of serotype 8, 11A and 33F is not reported to be universally high, [37,38,39] the pre-PCV-7 surveillance on IPD in England identified serotype 8 to be of highest invasiveness observed among capsular types causing disease in general population [40]. In

Massachusetts, post-PCV surveillance on IPD identified serotype 33F as highly invasive in children [38]. Strains of serotype 8 and 11A also significantly contribute to overall death toll from IPD with the risk of death associated with 11A being one of highest reported for any capsular type [41]. It remains to be determined in epidemiological studies to what degree ZmpC contributes to the overall burden of pneumococcal disease and to the emergence of certain non-vaccine serotypes in the carriage and in the disease after PCV-7 implementation.

Our results clearly show that ZmpC cleaves the P-selectin-interacting domain of PSGL-1, abrogating the binding of the functional-blocking mAbs, PL1 and KPL1, while leaving the epitope of the non-blocking mAb PL2 intact. PL1 binds to the segment of PSGL-1 spanning residues 49 through 62 [42] and KPL1 interacts with the tyrosine sulfation consensus motif from residue 48 to 52 [43]. The non-blocking antibody PL2 binds the residues 188 to 235, a membrane-proximal domain of PSGL-1 [43]. The ZmpC-mediated cleavage site on PSGL-1 is located between the epitopes of PL1, KPL1 and the non-blocking epitope of PL2, however the robust glycosylation of PSGL-1 hampered our attempts to determine the exact cleavage site by N-terminal sequencing. Unlike for the binding of PSGL-1 to P-selectin, tyrosine sulfation is not required for cleavage by ZmpC. Cleavage was however dependent on proper O-glycosylation, since treatment of leukocytes with sialidase completely restored the KPL1 mAb binding after treatment with ZmpC. *S. pneumoniae* itself produces up to three sialidases, NanA, NanB and NanC. Both NanA and NanB have a distinct role in pneumococcal virulence [44]. The removal of sialic acids from PSGL-1 by pneumococcal sialidases could potentially counteract the function of ZmpC, however, our experiments with concentrated supernatants clearly sh ow that in the putative presence of sialidases, PSGL-1 cleavage by ZmpC still occurs.

Zinc metalloproteases of *S. pneumoniae* are highly specific proteases, cleaving only one or a few host targets. They are characterized by the presence of a HEXXHE zinc-binding catalytic motif, however the specificity is most likely mediated by domains other than the common zinc-binding catalytic domain. IgA protease cleaves only the hinge region of IgA1 [45]. While the target for ZmpB is not yet identified, ZmpC is described to activate MMP9 [21]; however, the exact cleavage site and whether this occurs *in vivo* remains to be determined. Two proteoglycans, syndecan-1 and MUC-16, are shed by ZmpC from the membrane of epithelial cells [22,23]. These proteoglycans are highly O-linked glycosylated, which seems, as for PSGL-1, to contribute to the target specificity of ZmpC. However, other determinants, like primary amino acid sequences, must act in concert with O-linked glycosylation in determining the substrate specificity, as various heavily O-linked glycosylated receptors in our multi-screen mAb inhibition assay, for instance CD44, were not cleaved by ZmpC. Furthermore, the addition of human serum did not influence the cleavage rate, while containing multiple O-linked glycosylated proteins.

Our *in vivo* data clearly show less neutrophil extravasation towards the lungs in mice intranasally infected with wild type *S. pneumoniae* as compared to the isogenic ZmpC knockout strain. Eight hours after the onset of infection, a difference in bacterial clearance could not yet be detected. This is in line with Oggioni *et al*, who did not observe a difference in bacterial clearance 24 hours after infection using comparable TIGR4 and ΔZmpC/TIGR4 strains [21]. However, 3 to 4 days post infection they did observe a reduction of 75% in mortality for the ZmpC mutant compared to the wild type. In addition to PSGL-1, ZmpC is also described to target MUC-16, MMP9, and syndecan-1, which might also account for the increased

virulence of wild type *S. pneumoniae* [21,22,23]. Degradation of MMP9 and MUC-16 might lead to better colonization and dissemination of bacteria into the bloodstream. However, we do not foresee a role for these targets in the ZmpC-mediated phenotype, inhibition of neutrophil infiltration, we observed. ZmpC cleavage of syndecan-1 might, however, play an additional role in this phenotype. The cell surface heparan sulfate proteoglycan syndecan-1 displays chemokines, such as IL-8 on the endothelium cell surface, necessary for proper neutrophil transmigration [46]. Through syndecan-1 cleavage, ZmpC might release the cell surface bound chemokines and, thereby hamper neutrophil recruitment. Inhibition of neutrophil extravasation, by cleavage of PSGL-1 and/or syndecan-1 must occur in the blood vessel. As bacteremia was not detected 8 hours after infection, we propose that ZmpC is translocated from the lung alveoli into the blood vessel through other means. It is known that in early stages of acute inflammation increased microvascular permeability occurs [47], resulting in extravasation of plasma components into the alveoli [47]. It is feasible, that during pneumococcal pneumonia this could result in diffusion of ZmpC into the capillary vessels of the lungs [48]. Moreover, pneumococcal pneumoniae is haemorrhagic as a result of damage of the capillary vessels of the lungs by pneumolysin [49]. It is likely that this also allows ZmpC transfer to the blood stream. As marked congestion of the capillaries is typical during pneumococcal pneumonia, ZmpC reaching the capillaries is more likely to stay localized and as a result remains concentrated, enabling cleavage of PSGL-1 and/or other targets involved in neutrophil migration. Moreover, *S. pneumoniae* antigens can be detected in urine of patients diagnosed with pneumococcal pneumonia without bacteraemia. This strongly indicates that at least some antigens do reach the bloodstream during localized pneumococcal pneumonia [50]. Finally, we show the presence of antibodies directed against ZmpC in sera of healthy individuals, indicating that exposure to the antigen is common.

All four Zmp's of *S. pneumoniae* contain an LPNTG motif located near the N-terminus, next to the Gram-positive secretion signal. For IgA protease it was shown that this results in both its expression on the surface and secretion in the supernatant [51]. Although it is impossible to determine the levels of ZmpC during an infection *in vivo*, it was previously reported that Zmp's are the most abundantly produced proteases of *S. pneumoniae* [52]. In corroboration with these data, our purification of ZmpC yielded 250 ng/mL ZmpC under laboratory conditions, which is in line with Chen [22]. This concentration is at least 5 times higher than the determined IC_{50} for PSGL-1 cleavage (50 ng/mL). The concentrations measured *in vitro* are thus at least high enough to observe activity *in vivo.* Indeed, in our murine acute pneumonia model we observed that the deletion of ZmpC results in an enhanced neutrophil influx and less PSGL-1 removal from the surface of extravasated neutrophils, indicating that wild type strains produce enough ZmpC to exert its effect.

In conclusion, we report PSGL-1 as a novel target for ZmpC and add inhibition of neutrophil extravasation to the list of *in vivo* mechanism(s) by which ZmpC adds to the virulence of *S. pneumoniae*.

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Supplementary information

Mutant construction

The TIGR4 mutant strains were constructed by PCR ligation mutagenesis, creating gene replacements of the metalloproteinase genes with the Janus cassette containing the selectable markers *aphIII* (resistance to kanamycin) and counter-selective *rspL+* (dominant susceptible allel of *rpsL* gene) [1]. Primers were designed to amplify the 5'- and 3'-flanking regions of target genes *zmpC* (SP_0071), *zmpB* (SP_0664), and *Serine protease* (SP_0641) with genomic DNA from strain TIGR4 as template (Table S2). Flanking amplicons were 500–1200 bp in length. Restriction sites were integrated into the two primers nearest each target gene and into the Janus primers to allow for directional ligation as described[2]. After digestion of the amplicons, tripartite ligation of the flanking amplicons and the Janus cassette was carried out, and the ligation mix was used directly to transform TIGR4S as previously described [2]. Transformants were selected on blood agar supplemented with

kanamycin at concentration 100 mg/L. The expected replacement of each target gene with the Janus cassette by reciprocal recombination was confirmed in streptomycin-susceptible transformants by PCR. TIGR4*ΔZmpC* double cross over mutant and TIGR4*ΔZmpC* complemented strain were generated as follows; Flanking amplicons of ZmpC were blunt end ligated and amplified using Primers BS003 and BS008 (Table S2). For the TIGR4*ΔZmpC (+ZmpC)* strain this primer pair was used to amplify *zmpC* together with the flanking regions from genomic DNA from strain TIGR4. Both amplicons were used to transform competent TIGR4*ΔZmpC::Janus* and transformants selected by plating competence cultures on blood agar medium supplemented with streptomycin at concentration 200 mg/L. Streptomycin-resistant transformants were checked by PCR for the presence of expected size amplicon. Detection of *zmpC* in pneumococcal clinical isolates was performed on chromosomal DNA with primer pair BS033-BS034, resulting in a 577bp amplicon when the gene was present. Strains were selected to be screened for *zmpC* according to serotype distribution in IPD in the Netherlands. To explore further an association between serotype and *zmpC* number of serotype 11A and 33F isolates tested was arbitrarily increased after initial screening revealed *zmpC* presence in fewer isolates of these serotypes.

ZmpC isolation

To isolate ZmpC from TIGR4 culture, 4L of TIGR4*ΔzmpB* supernatant was collected and

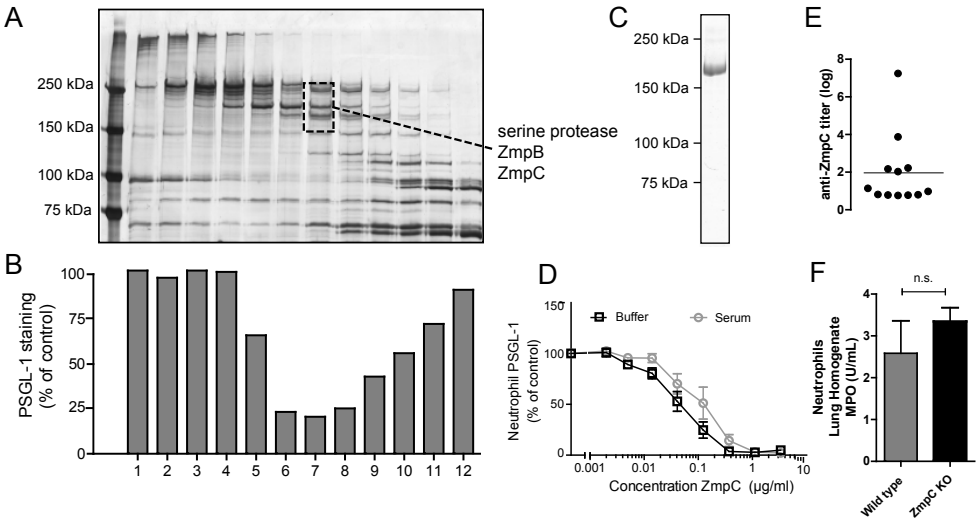


Figure S1. Purification of ZmpC from TIGR4 culture supernatant. Culture supernatant of TIGR4 was concentrated 200x with a 100 kDa cut-off amicon device and loaded on a Superdex 75 gel filtration column. (A) Collected fractions (0.5 mL) were separated by SDS-PAGE and the gel was silverstained. (B) Neutrophils were treated with the gel filtration fractions (1–12) for 30 min at 4°C. Cells were stained with PE-labeled anti-PSGL-1 (KPL1) for 30 min at 4°C. The percentage PSGL-1 staining was determined by comparison of mean fluorescence with untreated cells. The proteins of interest were identified by mass-spectrometry as: ZmpB, ZmpC and Serine protease of *Streptococcus pneumoniae.* (C) Purity of ZmpC after large-scale purification from TIGR4ΔZmpB culture supernatant analyzed by SDS-PAGE and Instant-blue staining. (D) The effect of 10% serum on PSGL-1 cleavage induced by ZmpC. PSGL-1 on human neutrophils was stained with PE-conjugated mAb KPL1 and measured by flow cytometry. The percentage PSGL-1 staining was determined by comparison of mean fluorescence with untreated cells. Data represent the mean ±s.e.m. of three independent experiments. (E) Anti-ZmpC IgG detected in the sera of 12 healthy volunteers. (F) Neutrophils in pulmonary interstitium measured by MPO activity in lung homogenates 8 hours after onset of disease.

secreted proteins were precipitated with ammonium sulfate (60% saturation) overnight at 4°C. Precipitated proteins were pelleted, dissolved in distilled water and dialyzed against 20 mM sodium phosphate pH 7.0 Sample was loaded on a DEAE column (GE Healthcare), washed with phosphate buffer and eluted with the same buffer + 2 M NaCl. Active fractions in the antibody competition assay were concentrated using a 100 kD cut-off ultrafiltration device (Millipore) and applied on a Superdex 200 (GE Healthcare) size exclusion column equilibrated with PBS. Active fractions were diluted 5 times in 20 mM phosphate buffer pH 7 and loaded on a MonoQ 5/50 GL column (GE Healthcare). After washing, proteins were eluted with a gradient of 0-0.5 M NaCl. Fractions were analyzed for purity using SDS-PAGE and Instant Blue (Expedeon) staining. Proteins of interest were identified by mass spectrometry. The protein concentration was determined with a standard BCA kit (Pierce).

Neutrophils in pulmonary interstitum

The infiltration of neutrophils into pulmonary interstitium was evaluated by the myeloperoxidase (MPO) (an intracellular enzyme specific for neutrophils) activity in lung homogenates that had undergone BAL. Lungs were homogenized in 50 mM potassium phosphate buffer (pH 6.5) and Triton X100 was added 0.05% (final concentration). After cellular lysis homogenates were cleared by centrifugation and hexadecyltrimethylammonium bromide was added to achieve a final concentration of 0.5%. MPO was evaluated by adding 20 µl of homogenate supernatant to180 µl of phosphate buffer, containing *o*-dianisidine at 100 µg/ml and hydrogen peroxide at 0.05%. Absorbance was read at 450 nm against a standard curve made with commercial MPO (Sigma).

Detection of ZmpC antibodies

Anti-ZmpC IgG was detected as described [3].

Supplementary Table 2 strains used in this study

Strain	Description	Source or reference
TIGR4		Tettelin et al Science
TIGR4S	TIGR4 but Sm^r by selection of mutants	Trzciński et al 2003
TIGR4Δ*ZmpB janus*	TIGR4s but transformed with ZmpB::kana:rpsL PCR product Kn^r Sm^s	this study
TIGR4Δ*SP janus*	TIGR4s but transformed with serine protease::kana:rpsL PCR product Kn^r Sm^s	this study
TIGR4Δ*ZmpC janus*	TIGR4s but transformed with ZmpC::kana:rpsL PCR product Kn^r Sm^s	this study
TIGR4Δ*ZmpC*	TIGR4:ZmpC but markerles by transformation with blunt end ligated ZmpC flanking regions; Kn^s Sm^r	this study
TIGR4Δ*ZmpC* (+*ZmpC*)	TIGR4:ZmpC but complemented by transformation with with ZmpC + flanking regions PCR product; Kn^s Sm^r	this study
TIGR4Δ*pspA*	TIGR4S but *pspA ::kan-rpsL*$^+$	Trzciński et al 2008

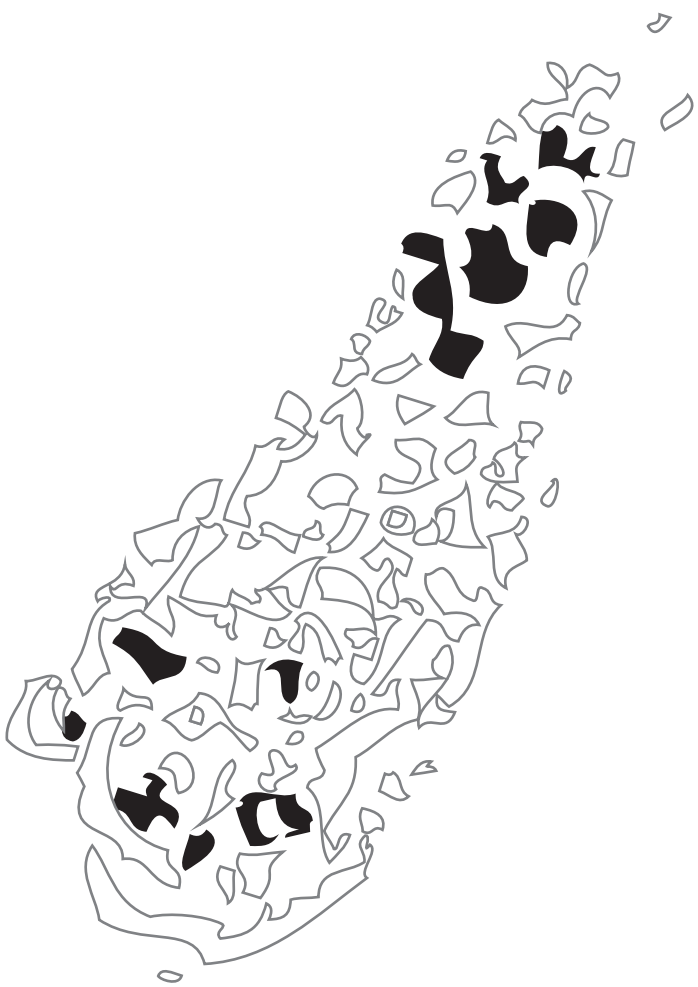
Supplementary table 2: Primers used in this study

Primer	Description	Reference
TTM051	CTTTGGGCAGTAGTGGTAAC; corresponds to position 853-834 upstream of*pspA* in TIGR4	
TTM059	GGTCTGATCCTTGCCATTGTC; corresponds to position 859-839 downstream of*pspA* in TIGR4	Trzciński et al 2008
BS003	AGTGACTGCCTCAAACCTTTCAACA; corresponds to position 959-935 upstream of*ZmpC* in TIGR4	this study
BS002	ATATGGATCCTCACCAATGCTTTTTCGGCTCA; position 11-32, corresponds to position 23-2 within o*ZmpC* in TIGR4	this study
BS007	ATATGGGCCCCGGATGAGGAGTCAGATGACAAATCGAC; position 12-39, corresponds to position 39-63 downstream o*ZmpC* in TIGR4	this study
BS008	TGGTAGTCCCCAGCTGCGATCC; corresponds to position 984-963 downstream of*ZmpC* in TIGR4	this study
BS015	GTTGAGGTTGAATTTATCAAGGCT corresponds to position 300-277 upstream of*ZmpB* in TIGR4	this study
BS011	ATATGGATCCAAAAGGCTTCCAAGAAATACAGAG; position 11-33, corresponds to position 1-23 upstream o*ZmpB* in TIGR4	this study
BS016	ATATGGGCCCATCTGCTTTGCGGATGAGGGTT; position 12-33, corresponds to position 13-34 downstream of*ZmpB* in TIGR4	this study
BS018	CTCCACGCGGTTGAGGCTCC; corresponds to position 988-968 downstream of*ZmpB* in TIGR4	this study
BS020	TGCAGAGATTGATTGTCTCGGCGT; corresponds to position 990-966 upstream of*SP* in TIGR4	this study
BS023	ATATGGATCCACATATTTATATAACTTCCAATAG; position 11-34, corresponds to position 12-35 upstream o*SP* in TIGR4	this study
BS025	ATATGGGCCCATTTATAACTCAATTATTTAGTT; position 12-35, corresponds to position 79-101 downstream o*SP* in TIGR4	this study
BS027	CCATACACACCAGAGATGAACCCCA; corresponds to position 939-914 downstream of*SP* in TIGR4	this study
BS033	AACCTTCACCAGTAAGACACGT; corresponds to position 2097-2117 of*ZmpC* in TIGR4	this study
BS034	CTTATCTCGATAGAGTTCAATGCC; corresponds to position 2672-2650 of*ZmpC* in TIGR4	this study

7

Summarizing Discussion

Common virulence mechanisms of *S. pneumonia* and *S. aureus*



In this thesis, the immunomodulatory properties of phenol-soluble modulins (PSMs) from *Staphylococcus aureus* and zinc metalloprotease C (ZmpC) from *Streptococcus pneumoniae* were investigated. This thesis elaborates on the function of these known virulence factors, and gives alternative, more comprehensive mechanisms and modes of action.

As described in **Chapter2**, humans are well equipped to defend themselves against bacteria. The innate immune system employs diverse mechanisms to recognize, control and if necessary destroy millions of different microbes. Only those bacteria able to escape detection or to counteract the sophisticated innate immune system can become pathogens. Both *S. pneumoniae* and *S. aureus* reside in the human upper respiratory tract having a rather commensal lifestyle. However, they can occasionally turn into pathogens causing a variety of potentially fatal diseases. Acute respiratory infections, in particular pneumonia, remain one of the most important causes of death in both adults and children, accounting for an annual estimated 3.5 million deaths worldwide [1]. Although carriage of these organisms is situated in the same niche, the upper respiratory tract, colonization of children with *S. pneumoniae* is negatively associated with *S. aureus* [2]. Competition is considered as the most common interaction between different organisms occupying the same niche, although host factors do contribute in creating an environment for the bacterium suitable to live. The main difference between the two pathogens is that the most common pneumococcal diseases do not contribute to pneumococcal transmission, suggesting that the virulence characteristics of the pneumococcus are probably adaptations that increase its persistence within a host during colonization. On the contrary, skin infections caused by community-associated methicillin-resistant *S. aureus* (CA-MRSA) have been shown to enhance transmissibility of the pathogen [3,4]. This difference probably can be explained by the multitude of immune evasion molecules and toxins for *S. aureus* to help in its virulence, as described in **Chapter 1**. However, *S. pneumoniae* has also developed mechanisms that modulate the host response and aid in its survival.

Capsule

Perhaps the best-studied virulence factor of *S. pneumoniae* is its capsular polysaccharide (CPS). Over 90 pneumococcal capsular serotypes exist, which differ in their polysaccharide chemistry and antigenicity. The capsule protects the pneumococcus against phagocytic clearance by blocking the deposition of immunoglobulins (Ig) and complement on the pneumococcal cell surface [5,6]. In addition, the capsule can decrease trapping by neutrophil extracellular traps (NETs) [7]. Strains that produce more capsule *in vitro* are more virulent *in vivo* [8], but the degree of encapsulation does not strongly impact nasopharyngeal colonization [9]. Although, some evidence exists for CPS in *S. aureus*, their contribution to virulence is not clear [10]. *S. aureus* capsules are only expressed in 40% of all circulating strains and the expression is limited to the stationary phase. Co-colonization of *S. aureus* and *S. pneumoniae* is negatively correlated, although they share the same niche. The anti-microbial properties described for PSMs might play a role in this phenomenon [11]. Almost all pneumococcal CPSs are negatively charged, which could increase their susceptibility for the positively charged highly amphipathic PSMs, similarly as described for human anti-microbial peptide HNP 1-3 [12].

Anti-opsonic properties

Next to CPS, the pneumococcus can express several surface proteins, which hamper opsonization. Although the mechanisms and proteins are different*, S. pneumoniae* has evolved similar ways of inhibition of opsonization, as described for *S. aureus* in **Chapter 1**. The pneumococcus can express IgA protease, allowing the specific cleavage of the most prominent Ig subclass in the human airway, thereby limiting the humoral response on mucosal surfaces [13,14]. Moreover, pneumococcal surface protein A (PspA) interferes with the deposition of C3 molecules on the pneumococcal cell wall, thereby inhibiting complement-mediated opsonization. In addition, PspC binds to factor H, which allows for the decay of complement convertases and thus prevents pneumococcal opsonization [15]. Compared to the vast arsenal of complement inhibitory proteins in *S. aureus* [16,17], the number of proteins involved in complement evasion in pneumococcus is small. One might speculate that putting enough energy into capsule biosynthesis is sufficient for protection against opsonophagocytosis. Nevertheless, both bacteria are very successful pathogens, both well-adapted to deal with host defenses.

Inhibition of neutrophil recruitment

Extracellular adherence protein (Eap), chemotaxis inhibiting protein of *S. aureus* (CHIPS), FPR2 inhibitory protein (FLIPr), its homologue FLIPr-L and staphylococcal superantigen-like 5 are all small secreted staphylococcal proteins that interfere with neutrophil activation or recruitment. These proteins are unique for *S. aureus.* In *S. pneumoniae* no comparable proteins targeting neutrophil recruitment have been found*.* However, in **Chapter 6,** we describe a new function for a known virulence molecule of *S. pneumoniae*. ZmpC targets the leukocyte adhesion molecule P-selectin glycoprotein ligand-1 (PSGL-1) and thereby hampers initial tethering and rolling of leukocytes on endothelial cells. Infection of mice with ZmpC-producing strain TIGR4 in the model of pneumococcal pneumoniae showed lower neutrophil infiltration into the lungs compared to animals infected with a genetic *zmpC* knock-out strain. The targeting of PSGL-1 seems to be a virulence mechanism shared by both *S. aureus* and *S. pneumoniae,* as also SSL5 antagonizes this receptor [18]. Additionally, Gram-negative bacteria secrete proteases that cleave multiple glycoproteins including PSGL-1 (e.g. ImpA of *Pseudomonas aeruginosa* and serine protease autotransporters of Enterobacteriaceae (SPATEs), thereby contributing to bacterial pathogenesis [19,20]. Except for ImpA, all these proteins targeting PSGL-1 are dependent on proper glycosylation of the receptor, as PSGL-1 treatment with neuraminidase diminishes their functional properties. The advantage of secreting proteases for the bacterium is that they can cleave multiple targets before inactivation, whereas antagonizing proteins can only inactivate a limited number of molecules. This might account for the difference in potency of SSL5 (IC_{50} = 3 µg/mL), compared to that of ZmpC (IC_{50} = 50 ng/mL) in biochemical assays. However, as proteases can have broad substrate specificities, determining the real biological significant targets may be difficult. Next to PSGL-1, human metalloprotease (MMP) 9, syndecan-1 and MUC-16 are other defined targets for ZmpC. Assessment of the relative contributions of each ZmpC target separately would only be possible by using genetically deleted mice.

Toxins

One common potent virulence strategy is the secretion of toxins or cytolysins. Pneumolysin (Ply) of *S. pneumoniae* is a cholesterol-dependent pore forming toxin, which is conserved in every pneumococcal isolate. Ply is a generally appreciated virulence factor and its contribution to disease was shown in multiple experimental models of infection [21]. In *in vivo* models of acute pneumonia, Ply was essential for the survival of *S. pneumoniae* in the respiratory tract [22]. Furthermore, Ply is required for bacterial dissemination from the lungs to other organs via the bloodstream [23] and immunization against Ply protects against *S. pneumoniae* infection [24]. Secretion of Ply by *S. pneumoniae* occurs solely upon autolysis, as Ply lacks a Gram-positive secretion signal. This seems to be quite an odd strategy, because the bacterium has to commit suicide before the toxin is released. However, pneumococcal strains deficient in either autolysin (LytA) or puryvate oxidase (SpxB) genes, involved in apoptotic-like death of pneumococcal cells, were out competed in an *in vivo* model of nasopharyngeal colonization, suggesting that release of virulence proteins from dead pneumococcal cells contributes to the ultimate survival of pneumococcus within the host [25].

S. aureus is characterized by the ability to secrete many toxins that damage the membranes of host cells. α-toxin, β-haemolysin, bi-component toxins, such as PVL, γ-haemolysins (HlgAB and HlgCB), leukocidin DE (LukDE), leukocidin GH (LukGH or LukAB) are all toxins that can lyse host cells. These toxins contribute to the development of abscesses or they kill neutrophils that are attempting to engulf and destroy bacteria [26]. Attempts have been made to characterize the role of each separate toxin in staphylococcal pathogenesis. However, these investigations are seriously hampered by the species specificity of the various toxins [27]. In addition, the production of most toxins is staphylococcal strain dependent. Even differences in growth conditions can give rise to conflicting results. Recently, the cell specificity of LukED was shown to be dependent on the G protein-coupled receptor CCR5 [28]. Based on this and many preliminary data from our own lab, it is tempting to say that the cell specificity or even species specificity for staphylococcal leukocidins is determined by the interaction with host G protein-coupled receptors.

Phenol-soluble modulins

This thesis elaborates on another class of staphylococcal toxins; the PSMs. PSMs are small cytolytic peptides only found in staphylococcus species. As shown in **Chapter 3**, these toxins have two main immunomodulatory functions: they attract phagocytes at nanomolar concentrations and are cytolytic at micromolar concentrations. Although PSMs can lyse multiple cell types and even liposomes, specificity for neutrophils in infection has been proposed, as these cells sense and migrate towards the PSM-producing bacteria [29,30]. One of the main findings of this thesis is the potent neutralization of PSMs by plasma lipoproteins. These findings have major implications for the current view on PSM function. Lipoproteins are highly abundant throughout the human body, as every cell depends on lipoproteins for their cholesterol and lipid metabolism. Lipoprotein-dependent neutralization of PSMs was shown *in vivo* in **Chapter 4**. However, more experimental infection models in HDL deficient mice are needed, in concert with using *psm* mutants, to make definite statements about the role of lipoprotein-dependent PSM neutralization and the function of PSMs in for example skin abscess formation and bacteremia.

Another functional aspect of PSMs is their property to attract neutrophils. This seems in contradition to other staphylococcal immune evasion molecules, such as CHIPS, FLIPr and FLIPr-L, which are employed to prevent the influx of neutrophils during certain stages of human colonization and infection. However, the expression of these immune evasion molecules is regulated under different conditions as compared to the PSM genes. PSM production, in contrast to CHIPS, FLIPr and FLIPr-L, is strictly controlled by the *agr* quorum-sensing system. FLIPr is expressed upon contact with neutrophil granule contents [31]. The *agr* system turns off most PSM expression at early stages of infection, when it may be advantageous for the bacteria to remain unrecognized by the innate immune system. FLIPr and FLIPr-L may help to inhibit the activity of any residual PSMs produced. At full blown infection, PSMs have the capacity of attracting neutrophils in the nanomolar range, exceeding their lytic capacity over a 1000 fold. Therefore, this mechanism is more likely to happen *in vivo* even in the presence of serum. In **Chapter 5**, we describe that *S. aureus* can actually survive inside neutrophils and that intracellular survival of *S. aureus* strain MW2 is dependent on PSM production. Higher PSM production inside the neutrophils after *S. aureus* phagocytosis may rescue CA-MRSA from phagosomal killing, contributing to its enhanced virulence. This is in line with previous findings where intracellular survival contributes to staphylococcal pathogenesis [32,33]. Thus, neutrophil attraction to the site of infection might be advantageous for the bacterium at certain stages of disease.

Analogous to PSMs, Ply has also been described to act pro-inflammatory. *S. pneumoniae,* expressing a mutated Ply protein lacking cytolytic activity, was shown to be more virulent than a pneumococcus in which the *ply* gene was deleted. This pro-inflammatory action was shown to be specific for TLR4, since the non-cytolytic mutant was reported to activate TLR4-dependent pathways [34]. These studies seem convincing, however, their findings may actually be artifacts, as was also the case for PSMs and many other proposed ligands for TLRs [35]. Real conclusive data on Ply may come from co-crystal structures, as shown for TLR4 in complex with MD2 and LPS.

Vaccination strategies

With the rise of antibiotic resistant strains of both *S. aureus* and *S. pneumoniae*, there is an urgent need for new classes of antibiotics. As an alternative strategy, vaccination is an attractive option, as it prevents the risk of infection. For pneumococci, well-defined polysaccharide vaccines are available, that allow efficient clearance of *S. pneumoniae* by the innate immune system. The most commonly used today consist of purified polysaccharides from 7 to 13 (conjugated CPS) or from 23 (pure polysaccharide) serotypes. Vaccination with pure polysaccharide vaccines is protective against invasive pneumococcal disease (IPD) in adults, whereas conjugated vaccines protect against disease and eradicate carriage in all age groups including infants [36,37]. The potential major drawback of CPS vaccines is the limited serotype coverage, as it may lead to replacement of the niche by strains not carried by the vaccine [38], highlighting the need for inclusion of additional CPS serotypes in the vaccine. Perhaps, inclusion of other surface and virulence factors such as pneumolysin or ZmpC (**Chapter 6**) might significantly boost the vaccine efficacy. Otherwise, it will be an enormous technical challenge to include CPS of all serotypes causing disease in humans in the vaccine. Nevertheless, this might eradicate pneumococcal disease.

By contrast, no vaccine is available for *S. aureus*. Although several attempts have been

made, most vaccine candidates failed in clinical trials, despite being protective against staphylococcal infection in animal models. One possible reason for this outcome is the lack of correlation between uptake of the bacteria by neutrophils and their subsequent destruction. Vaccination efficacy is highly dependent on the uptake and killing of *S. aureus* by neutrophils, however, as described in **Chapter 1 & 5**, staphylococcal isolates can survive inside neutrophils. *S. aureus* not only survives inside neutrophils, but also due to its ability to resist the effects of neutrophil-derived reactive oxygen species and AMPs. Also many other immune evasion factors , e.g. protein A, Sbi, SCIN, CHIPS, Efb, Ecb, FLIPr, FLIPr-L, and most of the SSLs, make vaccines against *S. aureus* less effective, as they hamper the attraction, complement or Ig opsonization, or prevent phagocytic uptake by neutrophils.

As an alternative or in combination with current vaccine strategies, targeting toxins and immune evasion molecules are undertaken. For Hla it has been shown that vaccination with the non-cytolytic Hla mutant prevents against murine pneumonia caused by CA-MRSA strains [39]. The same group showed that vaccination against non-toxigenic protein A raised antibodies that blocked the virulence of staphylococci, promoted opsonophagocytic clearance, and protected mice against challenge with highly virulent MRSA strains [40]. Strategies specifically targeting PSM peptides must take into account that these virulence factors exert a major function in pathogenesis within the neutrophil, which might make strategies employing anti-PSM antibodies impossible.

Concluding remarks

With worldwide problems of increasing resistance of *S. aureus* and *S. pneumoniae* to conventional antibiotics, new clinical approaches are highly needed. As discovered already in the 19th century, the neutrophil is a central player in the interplay between host and bacteria. This thesis describes multiple mechanisms by which Gram-positive pathogens such as *S. aureus* and *S. pneumoniae* successfully counteract the attack by neutrophils. A first striking feature is the potential of *S. aureus* to counteract the host response at nearly all levels, allowing the employment of its armament depending on local infection dynamics. The extensive quantity of strategies stress the importance of neutrophil escape in staphylococcal pathophysiology.

The need for new treatment options, both preventative and curative, is high. A better understanding of this biological war between pathogens and the neutrophil might offer leads for the development of new drugs and vaccines.

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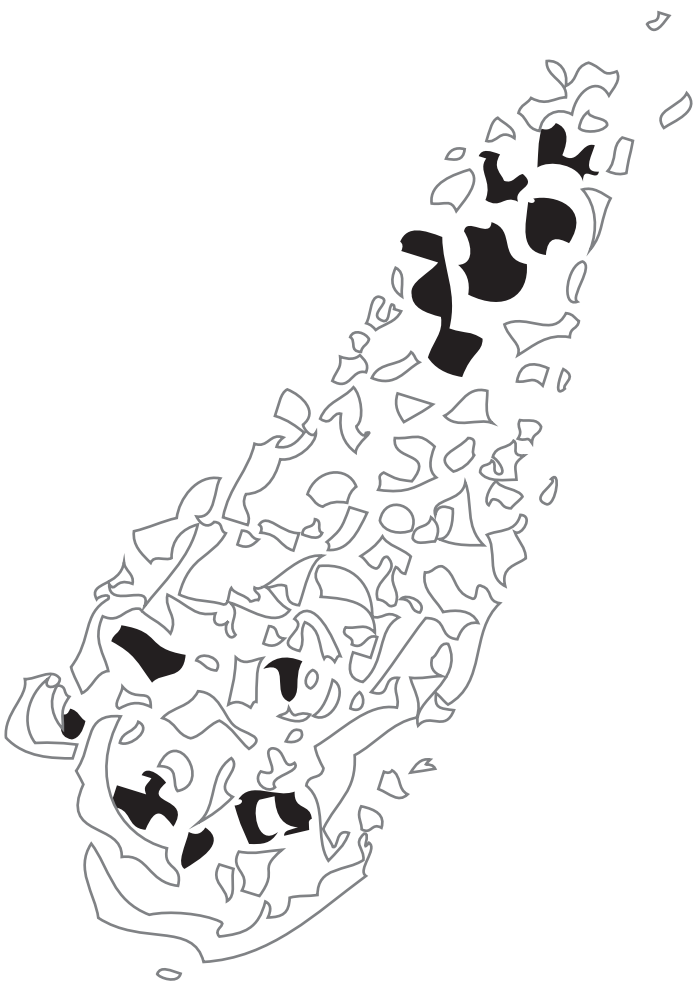
Nederlandse Samenvatting

voor niet ingewijden



De mens wordt continu blootgesteld aan miljoenen bacteriën. Deze bevinden zich op de huid, in je mond, neus en keelholte, en in je darmen. Alleen al in de darmen zit ongeveer 1 pond aan bacteriën en het aantal bacteriële cellen dat de gemiddelde mens met zich meedraagt is een tienvoud van alle menselijke cellen. Men kan dus eigenlijk concluderen dat de mens een lopende gastheer is voor miljarden verschillende bacteriën. Tegen bacteriën wordt meestal vies aangekeken, terwijl meer dan 99,9 % van alle bacteriën helemaal niet schadelijk of ziekteverwekkend is. Het merendeel van alle bacteriën die je met je meedraagt hebben juist een hele voordelige functie. Zo helpen bacteriën bijvoorbeeld in je darmen je met de vertering van verschillende voedingstoffen, die zonder deze bacteriën niet kunnen worden afgebroken en bezetten ze in de darmen de plaats van kwaadaardige bacteriën.

Dat de meeste bacteriën niet schadelijk zijn komt doordat ze heel efficiënt in toom worden gehouden door het menselijk immuunsysteem. De meeste microben worden getolereerd op plaatsen waar ze geen kwaad kunnen, zoals op de huid en op slijmvliezen. Maar ook als ze binnendringen is het meestal helemaal geen probleem. Witte bloedcellen en vooral neutrofiele granulocyten zijn in grote getallen aanwezig in de bloedbaan. Zodra er een bacterie binnendringt, wordt hij herkend (**hoofdstuk 2**) en verplaatsen de neutrofielen zich naar de plaats van infectie. Daar kunnen zij dan zonder al teveel moeite de bacterie opeten door een proces genaamd fagocytose. Na fagocytose gebruikt de neutrofiel een heel arsenaal aan wapens om de bacterie te vernietigen. Zo zit de neutrofiel propvol met blaasjes (granula) die gevuld zijn met afbraakenzymen en andere antimicrobiële stoffen. Deze granula worden na opname verenigd met de bacterie. Bovendien worden er allerlei zuurstofradicalen gevormd die heel erg giftig zijn voor bacteriën. De neutrofielen zijn dus heel belangrijk om de gezondheid van mensen te bewaken. Dit wordt nog duidelijker bij patiënten die door een genetisch defect een verminderde neutrofiel functie hebben. Deze patiënten worden continu geplaagd door terugkerende infecties van bacteriën.

Neutrofielen en andere factoren van het aangeboren immuunsysteem bewaren dus continu de rust in het lichaam en zorgen er voor dat vele miljoenen bacteriën die per ongeluk het lichaam binnen dringen worden opgeruimd. Echter, sommige bacteriën hebben allerlei strategieën ontwikkeld om juist deze afweermechanismen te omzeilen. Hierdoor kunnen zij juist beter overleven op plaatsen waar andere bacteriën snel worden opgeruimd. Dit worden pathogene bacteriën genoemd. In dit proefschrift is onderzoek gedaan naar de mechanismen van twee pathogene bacteriën: *Staphylococcus aureus,* letterlijk de “gouden druiventros” en *Streptococcus pneumoniae*, de pneumokok.

Staphylococcus aureus (*S. aureus*) komt voor bij ongeveer 30% van de mensen op de huid, in de neus, de keelholte en in de darmen, zonder dat zij daar enige hinder van ondervinden. Echter bij sommige mensen kan hij infecties veroorzaken variërend van onschuldige puistjes tot aan zware levensbedreigende aandoeningen als longontsteking (pneumonie), hersenvliesontsteking (meningitis), hartklepontsteking (endocarditis), botontsteking (osteomyelitis) en septische shock. *S. aureus* is vooral berucht als multiresistente bacterie; ongevoelig voor nagenoeg alle gangbare penicillineachtige antibiotica. Met name de methicilline resistente *S. aureus* (MRSA) zorgt -vooral in ziekenhuizen- wereldwijd voor grote problemen. Gelukkig vallen in Nederland de problemen door het strenge beleid en de strikte isolatiemaatregelen tot dusver erg mee.

Wat de meeste mensen echter niet weten is dat *S. aureus* en de mens in een wapenwedloop

verwikkeld zijn geraakt. Hoewel het menselijke immuunsysteem normaal gezien gelukkig de boventoon voert, heeft ook *S. aureus* zich tot de tanden bewapend om het immuunsysteem te ontmantelen. In **hoofdstuk 1** worden de verschillende strategieën van het immuunsysteem en de ontwijkingmechanismen van *S. aureus* beschreven.

Een van de belangrijkste strategieën van *S. aureus* is het maken van gifstoffen of toxines die de immuuncellen kunnen doden. In 2007 werd een nieuwe groep toxines in *S. aureus* ontdekt; de zogenaamde phenol-soluble modulins (PSMs). Sinds hun ontdekking zes jaar geleden werden deze PSMs uitgeroepen tot de belangrijkste MRSA-gifstof. Een aantal opmerkelijke eigenschappen werden in de wetenschappelijke literatuur aan PSMs toebedeeld. Zo kunnen PSMs namelijk zelf in minieme concentraties neutrofielen aantrekken en, als de concentratie hoog genoeg is, de neutrofiel doden. Verder werden uit belangrijke klinische stammen de PSMs genetisch verwijderd. Hierdoor ontstaat een stam die geen PSMs meer kan produceren, maar voor de rest identiek is aan de wild type moederstam. Deze zogenaamde PSM knock-outstammen werden gebruikt om aan te tonen dat PSMs ook daadwerkelijk belangrijk zijn in het infectieproces. In verschillende ziektemodellen met muizen en met konijnen is aangetoond dat stammen zonder PSMs minder goed kunnen infecteren of dat de infectie minder groot is dan met de wild-type stammen. Hierdoor kan men dus concluderen dat PSMs erg belangrijk zijn voor de overleving van *S. aureus*.

In **hoofdstuk 3** beschrijven wij dat PSM-toxines heel anders werken dan gedacht. Het lichaam kan de toxines namelijk neutraliseren in het bloed of serum. Serum is de vloeistof die overblijft als men bloed laat stollen en het stolsel afcentrifugeert. Wij hebben ontdekt dat PSMs worden geïnactiveerd door lipoproteïne deeltjes die normaal gesproken betrokken zijn bij het transport van vetachtige stoffen door het lichaam. Vet is niet oplosbaar in water en omdat cellen in het lichaam toch vetachtige stoffen nodig hebben, worden ze voor transport verpakt in lipoproteïne deeltjes. Door deze ontdekking hebben wij de tot dusverre gangbare hypothese dat: PSMs worden uitgescheiden en dan van buiten af de neutrofiel doden, verworpen. De hoeveelheid lipoproteïnes in het lichaam is namelijk zo groot dat onder normale omstandigheden in het bloed of in weefsels alle door de bacterie geproduceerde PSMs meteen geneutraliseerd worden. Lipoproteïne deeltjes kunnen worden onderscheiden op basis van dichtheid. HDL, LDL en VLDL vormen de belangrijkste groepen. Wij hebben ontdekt dat HDL de meeste belangrijke remmer van PSMs is. Er zijn zeer zeldzame erfelijke aandoeningen, zoals de ziekte van Tangier, waarbij patiënten geen zogenaamde HDL partikels kunnen vormen. **Hoofdstuk 4** laat zien dat het serum van deze mensen minder goed tegen PSMs beschermd dan dat van normale mensen. Verder zijn er muizen die ook geen HDL partikels kunnen vormen. Bij inspuiten van PSMs in deze HDL deficiënte muizen zien we dat PSMs ook minder geneutraliseerd worden en dus effectiever zijn.

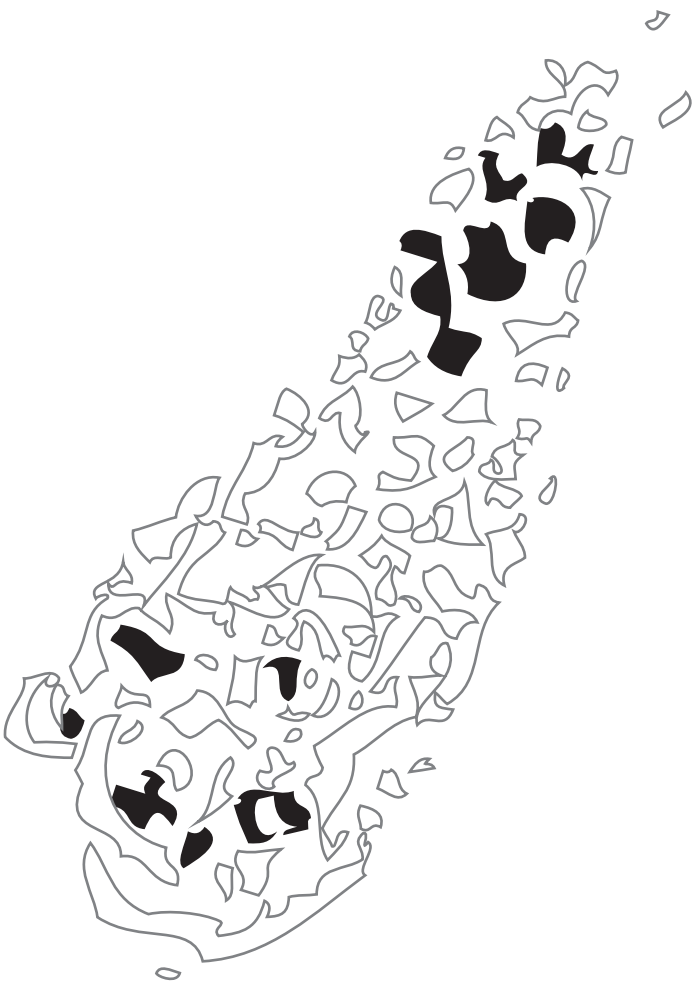
Maar PSMs zijn wel degelijk gevaarlijk, want in infectiemodellen spelen ze een cruciale rol! De PSM-toxines helpen de bacterie te overleven nadat ze gefagocyteerd zijn door neutrofielen. *S. aureus* die opgenomen is door een neutrofiel gaat heel veel PSM-toxines maken. In **hoofdstuk 5** laten we zien dat de neutrofiel daardoor sterft en de bacterie weer vrij komt. Vooral de kleinere alfa-type PSMs zijn hierbij van belang. Deze bevindingen kunnen misschien verklaren waarom er nog steeds geen goed vaccin tegen *S. aureus* is, terwijl hier wel heel lang naar gezocht wordt. Vaccinaties zijn vooral gebaseerd op het idee om bacteriën effectief te laten opnemen door neutrofielen. Maar wanneer ze dan daarna

weer kunnen ontsnappen hebben deze vaccinaties niet veel nut.

Een andere bacterie die voornamelijk bij kinderen en ouderen voor veel problemen zorgt, is *Streptococcus pneumoniae* of de pneumokok. De pneumokok is wereldwijd de belangrijkste veroorzaker van longontsteking, nekkramp en bloedvergiftiging bij kinderen. Deze bacterie beschermd zichzelf door middel van het maken van een kapsel van polysachariden. Tot dusver zijn er meer dan 90 verschillende kapsels beschreven en verreweg het meeste onderzoek wordt naar deze virulentiefactor gedaan. De pneumokok beschikt echter ook het vermogen om verschillende proteases te produceren. Proteases zijn eiwitten die andere eiwitten in stukken knippen en opruimen. Proteases kunnen ook een functie hebben als virulentiefactor. Zo is uit eerdere studies gebleken dat de zinc metallo protease C (ZmpC) in muizen een belangrijke rol speelt bij longontsteking. Echter, het exacte mechanisme was nog niet ontrafeld. In **hoofdstuk 6** laten wij zien dat ZmpC een specifieke receptor van neutrofielen afknipt, waardoor deze niet zo effectief meer naar de plaats van infectie kunnen migreren. Hierdoor heeft de bacterie meer kans om te overleven, doordat er minder neutrofielen naar de plaats van infectie kunnen komen.

In conclusie: bacteriën hebben vele manieren om het menselijk immuunsysteem te omzeilen, en zo te overleven in de mens. Dit proefschrift heeft bijgedragen aan het ontrafelen van mechanismen en hoe voor de mens belangrijke pathogene bacteriën ziekmakend kunnen zijn. Deze strategieën kunnen hopelijk door vervolgonderzoek worden aangepakt waardoor er minder doden zullen vallen als gevolg van infectieziekten. Verder legt het onderzoek bloot waar de zwakke plekken van het immuunsysteem zitten. Mogelijk kunnen we deze inzichten in ons voordeel gebruiken ter bestrijding van ziekten waarbij het immuunsysteem op hol is geslagen.

Dankwoord



Vandaag kan ik eindelijk het belangrijkste hoofdstuk van mijn proefschrift schrijven, het dankwoord. Na vele jaren van hard werken, liggen alle andere hoofdstukken al panklaar voor de drukker en is het AIO-zijn bijna voorbij. Nu ik hieraan terugdenk, is het vooral een hele mooie tijd geweest, waarvan ik enorm heb genoten. Bovendien heeft het mijn leven totaal veranderd, ik heb er namelijk mijn vriendin ontmoet. Geen enkel moment heb ik spijt gehad dat ik hieraan ben begonnen. Mijn periode bij de afdeling Medische Microbiologie had nooit zo mooi kunnen zijn zonder de juiste begeleiding, goede samenwerking, leuke collega's en goede vrienden. Vandaar dit woord van dank.

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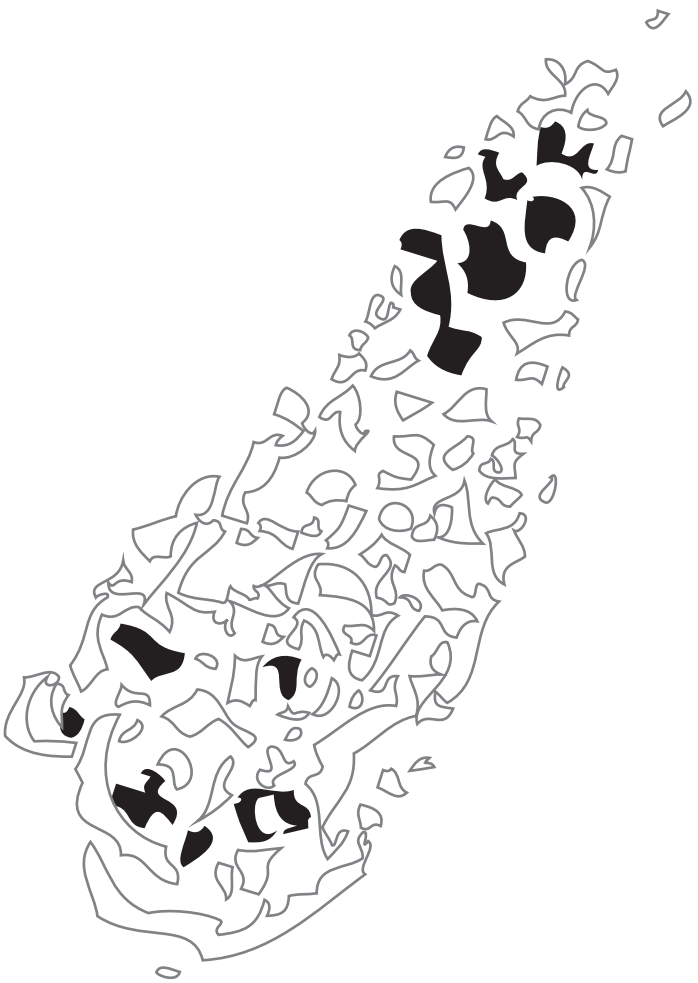
CurriculumVitea



Curriculum vitae

Bas Surewaard was born on March 12, 1984 in Breda, The Netherlands. He graduated from high school (Atheneum), Dongemond college, Raamsdonksveer in 2003. In the same year he started his study Biology at the University of Utrecht, and obtained his bachelor degree in 2006. During his master Immunity and Infection at the University of Utrecht, he performed his first internship under supervision of Dr. N.A. Georgiou and Prof.Dr. J.A.G. van Strijp at the department of Medical Microbiology at the University Medical Center Utrecht. His second internship was under supervision of Dr. H. van Eenennaam and C.L. Hofstra at the department of Target Discovery Immunology at (Organon) Schering/Plough in Oss, after which he graduated in 2009. In November 2008, he started his PhD training at the department of Medical Microbiology at the University Medical Center Utrecht under supervision of Prof.Dr. J.A.G. van Strijp and Dr. C.J.C de Haas. The results of this study are described in this thesis, and are published in different scientific journals. In June 2013, he will pursue his post-doctoral career at the Snyder Institute in Calgary, Canada. In the lab of Prof.Dr P. Kubes he will image immune evasion by bacteria *in vivo.*

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