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Functional basis for complement evasion by staphylococcal superantigen-like 7

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Summary

The human pathogen *Staphylococcus aureus* has a plethora of virulence factors that promote its colonization and survival in the host. Among such immune modulators are staphylococcal superantigen-like (SSL) proteins, comprising a family of 14 small, secreted molecules that seem to interfere with the host innate immune system. SSL7 has been described to bind immunoglobulin A (IgA) and complement C5, thereby inhibiting IgA-Fc α RI binding and serum killing of *E. coli*. As C5a generation, in contrast to C5b-9-mediated lysis, is crucial for immune defense against staphylococci, we investigated the impact of SSL7 on staphylococcal-induced C5a-mediated effects. Here, we show that SSL7 inhibits C5a generation induced by staphylococcal opsonization, slightly enhanced by its IgA-binding capacity. Moreover, we demonstrate a strong protective activity of SSL7 against staphylococcal clearance in human whole blood. SSL7 strongly inhibited the C5a-induced phagocytosis of *S. aureus* and oxidative burst in an in vitro whole blood inflammation model. Furthermore, we found that SSL7 affects all three pathways of complement activation and inhibits the cleavage of C5 by interference of its binding to C5 convertases. Finally, SSL7 effects were also demonstrated in vivo. In a murine model of immune complex peritonitis, SSL7 abrogated the C5a-driven influx of neutrophils in mouse peritoneum.

Keywords

Staphylococcus aureus; SSL7; complement evasion; C5a; IgA

Introduction

Cellular and humoral immune responses are important defense mechanisms that protect the host from invading pathogens and promote their clearance (Beutler, 2004). Complement is a major defense system that is initiated via three different pathways. The classical pathway is triggered by antibody-bound bacteria, while the lectin pathway specifically recognizes

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microbial sugar moieties. The alternative pathway is activated spontaneously or amplifies complement on bacteria previously opsonized via the classical and lectin pathways (Gasque, 2004; Walport, 2001). All pathways converge at the level of C3 by the formation of C3 convertases. The C3 convertases are responsible for the generation of C3b and iC3b, two components that mediate bacterial uptake by phagocytes via complement receptors. Incorporation of another C3b molecule into existing C3 convertases generates C5 convertases that cleave C5, resulting in the release of C5a and the sequential deposition of C5b-C9, leading to the formation of the so-called membrane attack complex (Pangburn and Rawal, 2002). This complex is responsible for direct killing of Gram-negative bacteria through osmotic lysis but is ineffective against Gram-positive bacteria that resist this response due to their thick peptidoglycan cell wall (Muller-Eberhard, 1986). The C5-cleavage product C5a is a proinflammatory molecule that attracts phagocytes to the site of infection, and primes them for bacterial uptake. Thus, generation of C5a is important for the clearance of Gram-positive bacteria (Mullaly and Kubes, 2006; Bohnsack *et al.*, 1997; Easmon and Glynn, 1976).

Staphylococcus aureus is an important human pathogen that causes community- as well as hospital-acquired infections. It is well-known for its suppurative diseases such as skin-limited abscesses and boils, but also causes endocarditis, pneumonia, sepsis, and toxic shock syndrome. Its pathology is linked to its arsenal of immune evasive molecules (Chavakis *et al.*, 2007). In addition to molecules targeting effector cells, *S. aureus* also secretes several small proteins that specifically interfere with the activity of the complement system (Rooijackers and van Strijp, 2007). Staphylococcal complement inhibitor (SCIN) targets C3 convertases (Rooijackers *et al.*, 2005), while extracellular fibrinogen binding (Efb) and extracellular complement binding (Ecb, also known as Ehp) proteins interact with C3b-containing convertases (Jongerius *et al.*, 2007; Hammel *et al.*, 2007). Thereby, these proteins effectively inhibit complement-mediated innate immune responses. The importance of interference with C5a-mediated responses was not only demonstrated for Ecb and Efb, but also for chemotaxis inhibitory protein of *S. aureus* (CHIPS) (de Haas *et al.*, 2004). CHIPS not only targets the C5a receptor (C5aR), but also binds the formyl peptide receptor (FPR). Thereby, CHIPS effectively blocks neutrophil chemotaxis towards C5a and formylated peptides. The staphylococcal superantigen-like (SSL) 7 binds immunoglobulin A (IgA) and complement component C5 (Langley *et al.*, 2005). The two-domain structure of SSL7 is homologous to superantigens, but it does not show superantigen activity (Al-Shangiti *et al.*, 2005). The N-terminal domain of SSL7 binds IgA and blocks IgA binding to the Fc α R (Ramsland *et al.*, 2007).

The binding of SSL7 to C5 has been shown to inhibit complement-mediated cell lysis of erythrocytes and *E. coli*, presumably by blocking the C5b-9 formation (Langley *et al.*, 2005). As SSL7 is produced by staphylococci that resist C5b-9, this study focused on the effects of SSL7 generation of C5a during opsonization of *S. aureus* and on complement-mediated clearance of staphylococci in vitro. We show a strong protective activity of SSL7 against staphylococcal clearance in human whole blood, independent of its IgA binding capacity. Finally, SSL7 also showed strong activity in vivo in a murine model of immune complex peritonitis.

Results

SSL7 inhibits C5a generation independent of IgA binding

Langley *et al.* (Langley *et al.*, 2005) have described that SSL7 binds C5 and inhibits serum lysis of *E. coli* and erythrocytes, most likely by preventing the cleavage of C5 and therefore the subsequent C5b-9-mediated cell lysis. However, C5b-9-mediated lysis does not play a role in the innate immune response to *S. aureus* as the thick peptidoglycan layer of the

bacterium protects it from lysis. On the other hand, the generation of C5a is important in the innate immune defense during staphylococcal infections. To investigate whether SSL7 production by *S. aureus* inhibits generation of C5a, we incubated 10% human serum with *S. aureus* for 30 min. This leads to the opsonization of the bacterium with C3b and generation of C5 convertases on the bacterial surface that cleave C5 into C5a and C5b. After pelleting of bacteria, the supernate was tested for its C5a content using a calcium mobilization assay with U937 cells expressing the C5aR. Serum alone did not induce a calcium mobilization, whereas a clear response was observed when bacteria were added to the serum (Fig. 1A). Addition of SSL7 during bacterial opsonization completely inhibited this C5a-mediated response. In contrast, SSL7 could not inhibit the C5a-mediated response in U937-C5aR cells when it was added to the supernate after bacterial opsonization (Fig. 1A). This indicates that SSL7 acts during bacterial opsonization by binding C5 and does not bind and inhibit C5a itself nor its cellular C5aR. Figure 1B shows the same results with a concentration range of SSL7. Already 150 nM (equals 3 µg/ml) of SSL7, added during bacterial opsonization, could completely inhibit the C5a-mediated response. To investigate the specificity of SSL7 in this response, we also tested SSL10 in this assay. This staphylococcal protein is highly homologous to SSL7 and binds the chemokine receptor CXCR4 (Walenkamp *et al.*, 2009). In contrast to SSL7, SSL10 did not affect the C5a generation (Fig. 1B). Thus, SSL7 prevents cleavage of C5 and thereby abrogates the generation of C5a during staphylococcal opsonization by complement.

In addition to C5 binding, SSL7 is described to bind IgA (Langley *et al.*, 2005). The interaction with IgA may be of importance in its ability to prevent the cleavage of C5. To examine whether IgA contributes to the C5-inhibitory activity of SSL7, we determined the generation of C5a in an environment lacking IgA. For this purpose, *S. aureus* was first incubated with human serum for full opsonization. Subsequently, the bacteria were washed extensively and surface-bound C2a and Bb of the generated C3 and C5 convertases were allowed to dissociate to obtain C3b-covered bacteria. After washing, soluble purified C3, factor B, factor D, and properdin were added. Factor D cleaves factor B into activate Bb that interacts with deposited C3b to generate cell-bound C5 convertases, which are stabilized by properdin. After addition of C5 in the presence or absence of SSL7, the generation of C5a by the C5 convertases was evaluated by testing the supernates using the C5a-mediated calcium mobilization in U937-C5aR cells. Under these conditions, in which IgA was not present, SSL7 still effectively inhibited the generation of C5a (Fig. 1C), although its effect was somewhat reduced as compared to Fig. 1B.

To further evaluate the requirement of IgA for SSL7-induced C5-inhibition, an SSL7 variant lacking IgA-binding capacity was constructed. SSL7 mutants with the single point mutations L79A, N38T, and P82A are described to have 91-, 35-, and 35-fold reduced IgA affinities, respectively (Ramslund *et al.*, 2007). Here, we made a mutant of SSL7 with all three single mutations (SSL7-IgA mutant). This SSL7-IgA mutant did not bind IgA1 or IgA2 (Fig. 2A,B), but retained full C5-binding capacity (Fig. 2C), as compared to the wild type SSL7 protein. The effect of the SSL7-IgA mutant was tested in the *S. aureus* serum opsonization assay. In this case, C5a generated in the supernate after opsonization of the bacteria was examined by Western blotting. As depicted in Figure 2D, C5a could clearly be detected in the supernate after opsonization of *S. aureus* with serum, in contrast to serum alone. Addition of SSL7 as well as SSL7-IgA mutant to serum during opsonization resulted in a clear inhibition of C5a generation. Therefore, these results demonstrate that SSL7 does not require IgA binding for its C5-inhibitory effect.

SSL7 inhibits the C5a-dependent clearance of *S. aureus* in an in vitro whole blood inflammation model

The generation of C5a after staphylococcal opsonization is crucial for a proper innate immune response to the bacteria. First, C5a induces a chemotactic gradient for attraction of neutrophils to the site of infection. Next, it activates neutrophils for enhanced phagocytosis and oxidative burst leading to proper clearance of the bacteria. Using a C5a-dependent whole blood inflammation model (Rooijackers *et al.*, 2006; Mollnes *et al.*, 2002), we tested the effect of SSL7 on C5a-mediated phagocytosis of FITC-labeled *S. aureus*. In the absence of SSL7, we observed strong phagocytosis of staphylococci by neutrophils (Fig. 3A). When phagocytosis was measured with SSL7 or SSL7-IgA mutant added to whole blood, both proteins dose-dependently inhibited phagocytosis of staphylococci by neutrophils (Fig. 3A,B). Wild type SSL7, still able to bind IgA, showed a higher activity than the SSL7-IgA mutant. Subsequently, the oxidative burst was determined upon addition of bacteria to whole blood by measuring the reactive oxygen species generated in response to bacterial recognition. SSL7 also clearly inhibited the oxidative burst induced by *S. aureus* (Fig. 3C). Neither the *S. aureus*-induced oxidative burst nor the phagocytosis was affected by SSL10 under the same conditions (Fig. 3A,C), demonstrating SSL7-specific effects. These experiments indicate a strong protective activity of SSL7 against staphylococcal clearance in human whole blood.

SSL7 prevents binding of C5 to C5 convertases

By binding C5, SSL7 could block the generation of C5a through two different mechanisms. SSL7 may prevent the recognition of C5 by C5 convertases, or it may allow for the recognition and binding by C5 convertases but block its subsequent cleavage. Two different C5 convertases exist. C4b2aC3b is the C5 convertase produced upon activation of the classical and lectin pathway of complement, whereas (C3b)₂Bb is the C5 convertase of the alternative pathway. Thusfar, the effect of SSL7 on these separate complement pathways, which are all activated upon staphylococcal opsonization, is not known. To gain more insight into the inhibitory mechanism of SSL7, we examined its effect on all three separate complement pathways. Therefore, ELISA-based experiments were performed that examine all three pathways of complement activation individually at the C3 and C5 level by detection with antibodies directed against C3c and C5b-9, respectively. As expected, SSL7 did not affect complement activation when measured at the C3 level (Fig. 4A), but it effectively inhibited all complement pathways when the effects were measured at the C5 level of complement activation (Fig. 4B). This suggests that SSL7 activity is not dependent on one of the two C5 convertases.

To further address the mechanism of action of SSL7, we determined whether SSL7 could interfere with C5 binding to C5 convertases. For this purpose an ELISA-based experiment was set up using the alternative pathway of complement activation to generate a (C3b)₂ surface. For that reason, wells were coated with LPS, and the alternative complement pathway was activated with 30% serum in the presence of EGTA and MgCl₂, resulting in the deposition of (C3b)₂Bb complexes. The covalently-bound C3b subunits of these C5 convertases are responsible for C5 binding, whereas the non-covalently bound Bb molecules mediate the cleavage of C5. As C5 convertases are very unstable due to the dissociation of the Bb molecule, a sole (C3b)₂ surface was generated by allowing Bb molecules to dissociate for 30 min. The created surface enables the analysis of binding the substrate C5 without its cleavage by Bb (Wiesmann *et al.*, 2006). Figure 4C shows that C5 binding to C5 convertases is successful and specific as CR1g, which recognizes C3b molecules, abrogated C5 binding. SSL7 alone inhibited the binding of C5 to the C5 convertases for about 50%, whereas addition of IgA increased the SSL7 activity. Thus, the basis for SSL7-mediated

inhibition of C5a generation seems to reside in its effect on C5 binding to C5 convertases. In this effect on C5 binding, IgA increases the SSL7 activity.

SSL7 inhibits complement activation in vivo in a murine inflammatory model

To determine SSL7 effects in vivo, we explored SSL7 activity in a relevant mouse disease model. First, the interaction of SSL7 with the mouse complement system was investigated. Previous studies already demonstrated that SSL7 binding to C5 was not human specific (Langley *et al.*, 2005); SSL7 is reported to bind C5 from human, chimpanzee, baboon, sheep, pig, rabbit, and goat serum, while binding to mouse, cow, and horse C5 could not be observed. Nevertheless, we assessed the effects of SSL7 on complement activation in mouse serum through a functional assay that examines the classical pathway-mediated hemolysis. Herein, opsonization of erythrocytes with antibodies prior to incubation with serum leads to complement-mediated lysis. In human serum, SSL7 inhibited the complement-mediated hemolysis (Fig. 5A). SSL7 also effectively blocked hemolytic complement activation in mouse serum (Fig. 5B). To further characterize the effect of SSL7 on mouse C5 cleavage, C5a generation after bacterial opsonization was measured. Addition of SSL7 during bacterial opsonization in mouse serum effectively blocked the generation of C5a (Fig. 5C). SSL7 thus interacts with mouse complement C5, and its functional activity cannot only be measured in human immune assays but also in mouse models.

Then, to assess the in vivo activity of SSL7, we used the well established model of immune complex-mediated peritonitis (Heller *et al.*, 1999a). In this model, formation of immune complexes in the peritoneum initiates the activation of the classical and alternative pathway of complement. This not only leads to edema and hemorrhage, but also to infiltration of neutrophils to the site of injection. Six hours after in vivo challenge with immune complexes in the peritoneal cavity, neutrophils infiltrated the mouse peritoneum (Fig. 5D). When mice were injected with SSL7 prior to challenge with immune complexes, we observed a complete inhibition of neutrophil accumulation in the peritoneum. Administration of SSL7 did not induce any neutrophil influx into the peritoneum. In conclusion, SSL7 inhibits complement-induced neutrophil influx in vivo.

Discussion

Staphylococcus aureus secretes several virulence factors to modulate immune responses and thereby escapes recognition and clearance by the host (Chavakis *et al.*, 2007). In this paper we demonstrate that the C5-binding protein SSL7 inhibits complement-mediated processes important for staphylococcal clearance. In an ex vivo whole human blood inflammatory model, SSL7 not only inhibited the generation of reactive oxygen species by phagocytes in response to staphylococci but also affected staphylococcal uptake by phagocytes. Previous experiments with this whole blood model demonstrated that the complement-mediated *S. aureus*- and *E. coli*-induced oxidative burst heavily depends on the generation of C5a and the C5a-C5aR interaction (Rooijackers *et al.*, 2006; Mollnes *et al.*, 2002). Furthermore, this study is the first to provide evidence for the anti-inflammatory activity of SSLs in an animal model.

Human C5 is a glycoprotein of 196 kDa that is secreted by the liver into plasma (Fredslund *et al.*, 2008; Fernandez and Hugli, 1978). It comprises an α -chain and a β -chain that are linked by a disulfide bridge. C5 convertases cleave C5 in the α -chain at Arg751-Leu752 to generate the small C5a of 8.2 kDa and the large C5b, which consists of the β -chain and a large part of the α -chain. SSL7 binds C5 and was described to prevent the C5b-9-mediated lysis of *E. coli* and human erythrocytes (Langley *et al.*, 2005). SSL7 not only targets C5 but concomitantly binds IgA. Both binding of serum and mucosal IgA is described, and SSL7 thereby prevents the interaction of IgA with its receptor Fc α RI (Langley *et al.*, 2005). Co-

crystallization of SSL7 and IgA demonstrated that SSL7 targets the C2 α /C3 α interface of IgA, which is also recognized by the Fc α RI (Ramsland *et al.*, 2007). Since IgA is found abundantly in the mucosa, SSL7 may be beneficial during colonization by *S. aureus*. Here, we demonstrate that SSL7 clearly prevents the generation of C5a. Although binding of IgA per se is not needed for the C5 inhibition by SSL7, it clearly adds to its activity. In both the C5a generation assay without IgA as in the phagocytosis assay using the SSL7-IgA mutant, less C5-inhibiting activity was found than in situations where IgA was present or wild type SSL7 was used. The same was true in the C5-C5 convertase binding assay, in which IgA increased the activity of SSL7. Therefore, we conclude that SSL7, with additional help of IgA, prevents C5 activation by inhibiting the binding of C5 to C5 convertases. We have tried to investigate the effect of SSL7 on C5 binding to the C5 convertase of the classical and alternative pathway (C4b2aC3b) as well but have failed by lack of specificity in this assay. During the reviewing process a publication by Laursen *et al.* of the crystal structure of the SSL7-C5 complex appeared showing that SSL7 binds to C5 far from the C5a binding site (Laursen *et al.*, 2010). They observed a slightly diminished activity of their SSL7-IgA mutant on C5a generation, comparable to our results. Therefore, they proposed that IgA most likely adds to C5-cleavage inhibition through steric hindrance. Here, we show that SSL7 does not seem to inhibit C5 cleavage but rather inhibits C5 binding to C5 convertases. Indeed, as suggested by Laursen *et al.*, IgA binding possibly aids in the inhibition by steric hindrance, as binding of IgA makes the C5-SSL7 complex more than twice as large.

Previous experiments pertaining to species specificity of SSL7 demonstrated that this protein was not human specific but also binds C5 of several other species (Langley *et al.*, 2005). Binding to murine C5, however, was not observed. In our *in vitro* and *in vivo* mouse models, SSL7 did impede the C5-driven processes. It is well-documented that mice often show genetic deficiency of C5. In fact, up to 39% of common inbred murine strains carry this deficiency and lack detectable C5 (Cinader *et al.*, 1964). The initial study describing the inability of SSL7 to interact with murine C5 did not document the mouse strain used for serum isolation. In our experiments, we made use of BALB/c serum and BALB/c mice, which express functional C5 (Cinader *et al.*, 1964).

To test the *in vivo* activity of SSL7 we selected an inflammatory model that is highly C5a-dependent. In the Arthus reaction, the formation of immune complexes propagates an immune reaction by complement activation. Hereby, C5a and the C5aR are essential effectors in the observed complement-mediated inflammatory reactions (Godau *et al.*, 2004; Heller *et al.*, 1999b). Administration of SSL7 fully blocked neutrophil influx into the mouse peritoneum in this model. SSL7 is therefore highly anti-inflammatory in this model of immune complex disease. Although C5a acts as a proinflammatory signal, aberrant expression has been implicated in several chronic and acute diseases such as stroke, reperfusion/ischemia, transplant rejection, rheumatoid arthritis, and tumor progression (Ricklin and Lambris, 2007). SSL7 thus may have a therapeutic potential as an anti-inflammatory compound in a variety of diseases and disease states. It must be noted however that, because of its bacterial origin, SSL7 is immunogenic, and identification of mimics that are less immunogenic but retain the same specificity and activity would make it more suitable as therapeutic agent.

Since C5 plays a role in bacterial clearance once the bacterium reaches the host tissue, C5-binding SSL7 probably plays an important role during the colonization and infection of *S. aureus*. The presence of antibodies directed against SSL7 in human serum (Arcus *et al.*, 2002) indicates production of SSL7 by the bacterium when it gets into contact with the host. SaPI2 encoding the main *ssl* cluster is present in all clinical *S. aureus* isolates, but its individual gene composition is variable (Fitzgerald *et al.*, 2003). Analysis of many *S. aureus* isolates from sheep, cows, and poultry demonstrated that this cluster is also present in all the

animal isolates tested so far. For SSL7 specifically, it is described that the gene encoding for this protein is found in 90% of clinical isolates (Monecke *et al.*, 2008) and is present in all of the tested animal isolates from chicken, sheep, goat, rabbit, and camel (Shuiep *et al.*, 2008; Smyth *et al.*, 2007). The high prevalence of *ssl7* in *S. aureus* strains and the production of SSL7 following host contact strongly suggest a beneficial role for SSL7 in bacterial survival within the host environment.

In addition to SSL7, *S. aureus* secretes several other complement inhibitors (Jongerijs *et al.*, 2007; Rooijackers and van Strijp, 2007). SCIN binds C3 convertases and inhibits the cleavage of C3 and all downstream effector functions (Rooijackers *et al.*, 2005). We have recently described that extracellular complement-binding protein (Ecb) blocks C3b-containing convertases and also effectively inhibits C5a-mediated responses in the mouse model used in this study (Jongerijs *et al.*, 2007). Moreover, *S. aureus* produces CHIPS, a potent C5aR inhibitor (de Haas *et al.*, 2004). Thus, staphylococci have generated a diverse, synergistic system of immune inhibitors that specifically target the complement system. The fact that so many staphylococcal proteins prevent C5a-mediated responses highlights the critical role for C5a in *S. aureus* killing. The importance of C5a in *S. aureus* infections has been demonstrated by Mullaly *et al.* (Mullaly and Kubes, 2006), showing that challenging of mice with live *S. aureus* in the presence of anti-C5aR antibodies results in significant increase in morbidity as compared to untreated mice. In addition, relevance of C5/C5a during *S. aureus* infection has been demonstrated in several other mouse models (von Köckritz-Blickwede *et al.*, 2009; Hopken *et al.*, 1996; Cerquetti *et al.*, 1983). The production of many complement inhibitors though complicates the use of an infection model. Therefore, we have chosen to test SSL7 activity in a mouse model that is fully dependent on the cleavage of C5a from C5. Currently, several *S. aureus* knock-out strains are constructed for future infection studies. These will provide us with a better understanding of the individual or joint contributions of the distinct staphylococcal C5a inhibitors.

Experimental procedures

Cloning, expression and purification of SSL7

The genes encoding for SSL7 and SSL10 were cloned from *S. aureus* strain NCTC8325. SSL10 was cloned and expressed as described previously for SSL5 (Bestebroer *et al.*, 2007). For the expression of SSL7 lacking a histidine tag, *ssl7* was cloned into the expression vector pRSETB (Invitrogen) using the NdeI and BglII restriction sites. SSL7 with L79A, N38T, and P82A point mutations (SSL7-IgA mutant) was generated using overlap extension PCR and cloned in the same way as SSL7. Amplified products were verified by sequencing. SSL7 and SSL7-IgA mutant were expressed in Rosetta-Gami(DE3)pLysS *E. coli* according to the manufacturer's protocol (Novagen, Darmstadt, Germany). The expressed SSL7 proteins were purified from inclusion bodies using CelLytic B Cell Lysis Reagent (Sigma), according to the manufacturer's description. Shortly, bacterial cells were lysed in CelLytic B Cell Lysis Reagent containing EDTA-free protease inhibitor cocktail tablets (Roche Diagnostics), 200 µg/ml lysozym, 20 µg/ml DNase and RNase (Roche), and 1.25 mM EDTA. After lysis, the inclusion bodies were pelleted down and washed twice with 0.5% LDAO (lauryl dimethyldodecylamine N-oxide; Sigma). Then, the inclusion bodies were dissolved in 50 mM Tris, 6 M Guanidine pH 10. The isolated SSL7 proteins were properly refolded by dialysis in 50 mM Tris pH 10 and pH 9 for 24 h and 16 h, respectively. Finally, the pH of the dialysate was lowered in 0.2 steps, of each 2 h, to reach pH 8. Then SSL7 was rebuffered to 20 mM sodiumphosphate pH 7 for further purification using ion exchange chromatography on a HiTrap-SP XL column. Finally, all isolated proteins were rebuffered to PBS and estimated to be more than 95% pure when assayed on SDS-polyacrylamide gel electrophoresis.

C5a generation

Generation of C5a during opsonization was performed with laboratory *S. aureus* strain Wood. Bacteria were grown in Luria Bertani (LB) medium to an OD_{660nm} of 0.5 and heat killed for 30 minutes at 70°C. Bacteria (1×10^7) were incubated with 10 % normal human serum (NHS) or fresh 30% normal mouse serum from CD-1 mice (NMS) in presence or absence of SSL7 or SSL7-IgA mutant for 30 minutes at 37°C (total volume: 100 µl). The bacteria were spun down, and the supernates were tested for presence of C5a by supernate-induced calcium-mobilization of U937-C5aR cells (a generous gift from Dr. E. Prossnitz, University of New Mexico, Albuquerque, NM). When indicated, SSL7 was added to the spun-down supernates after opsonization. For calcium mobilization, U937-C5aR cells were labeled with 2 µM Fluo-3-AM, a fluorescent probe for free intracellular calcium. Calcium mobilization was measured with a flow cytometer (FACSCalibur; Becton Dickinson, Franklin Lanes, NJ) for 10 seconds before and 40 seconds after stimulation with diluted supernates. Stimulation was determined at 5 seconds after stimulation and is expressed as the relative calcium increase of SSL7-treated cells compared to untreated cells.

Supernates were also tested for presence of C5a by analyzing the samples by SDS-PAGE and Western blotting. C5a was detected by staining the blots with polyclonal anti-C5a (Calbiochem, San Diego, CA) and goat anti-rabbit-HRP (Southern Biotechnology, Birmingham, AL).

To assess the importance of IgA binding to SSL7, C5a generation via bacterial opsonization was also tested in an environment deficient of IgA. Therefore, cleavage of purified C5 was assessed by bacterium-bound C5 convertases. To prepare C3b-covered bacteria, *S. aureus* was incubated with 20% human sera for 30 min in HBS²⁺ (Hepes-buffered saline, 20 mM Hepes, 140 mM NaCl, 5 mM CaCl₂, and 2.5 mM MgCl₂). After extensive washing, surface-bound C2a/Bb were allowed to dissociate for 30 min in PBS. To create C5 convertases, C3b-covered bacteria were incubated with 100 µg/ml fB, 0.75 µg/ml fD, and 4 µg/ml properdin (Calbiochem) in HBS²⁺ for 30 min at 37°C. C5 cleavage was assessed by adding 25 µg/ml C5 (Calbiochem) in the presence or absence of SSL7. Formation of C5a was measured by analyzing supernates in a calcium mobilization assay as described above.

ELISAs

Complement ELISAs were performed as previously described (Jongerijs *et al.*, 2007). Briefly, the lectin pathway was assessed by using immobilized mannan (*Saccharomyces cerevisiae*, Sigma) as a ligand, while LPS (*Salmonella enteritidis*, Sigma) and human IgM (Calbiochem) were used for the classical and alternative pathways, respectively. Plates were blocked with 4% BSA/0.05% Tween-20/PBS. Then, serum samples were added for 1h at 37°C. Serum samples were diluted in a buffer containing 20 mM Hepes, 140 mM NaCl, 0.1% gelatin (pH 7.4) to which 5 mM CaCl₂ and 2.5 mM MgCl₂ was added to assess the lectin and classical pathway, while 5 mM MgCl₂ and 10 mM EGTA were added for the alternative pathway. To evaluate SSL7 effect, serum samples were preincubated with SSL7 for 15 minutes at room temperature before adding to the plates. Finally, deposited C3b and C5b-9 were detected using anti-C3c WM1 (ATCC) and anti-C5b-9 (Abcam) antibodies, respectively, followed by peroxidase (PO)-conjugated goat anti-mouse IgG (Southern Biotechnology Associates).

For the analysis of C5 binding to C5 convertases, LPS (20 µg/ml, *Salmonella enteritidis*, Sigma) was adsorbed to a microtitre plate overnight at 4°C. After blocking with 4% BSA/PBS for 1 h at 37°C, 30% human serum in 20 mM Hepes buffer containing 10 mM EGTA and 5 mM MgCl₂ was added for one hour at 37°C for alternative pathway complement deposition. This was repeated with freshly prepared 30% human serum under the same

conditions for an additional hour. Surface-bound Bb molecules were allowed to dissociate for 30 min in PBS. A dilution series of C5 was added in the presence or absence of 10 µg/ml SSL7 (430 nM), SSL7 (430 nM) and IgA (60µg/ml) or CRIg (Complement Receptor of the Immunoglobulin superfamily, CRIg-S, Genentech) for 1 h at 37°C. Bound C5 was detected using anti-C5/C5a (Hycult biotechnology, The Netherlands) and peroxidase (PO)-conjugated goat anti-mouse IgG.

To study the binding of SSL7 to C5, IgA₁, and IgA₂, wells were coated with 430 nM SSL7 or SSL7-IgA mutant and incubated with a dilution series of C5, IgA₁, or IgA₂ (human anti-sheep erythrocyte immunoglobulin A (Boel *et al.*, 2000). Binding of C5 was detected with anti-C5a (Calbiochem) and goat anti-rabbit IgG-PO (Southern Biotechnologies) and IgA₁ and IgA₂ with sheep anti-human IgA-PO (ICN Biomedicals).

Phagocytosis

S. aureus was first labeled with FITC by incubating an exponential growth culture with 100 µg/ml FITC for 1 hour at 37°C in 0.1 M carbonate buffer (pH 9.6). Freshly isolated human blood was anticoagulated using 50 µg/ml lepirudin (Refludan, Schering, Kenilworth, NJ). Phagocytosis was performed by incubating FITC-labeled staphylococci (1×10^8) with 50% blood for 25 minutes at 37°C (total volume: 200 µl) under vigorous shaking. The cells were fixed and erythrocytes lysed by addition of BD FACS lysing solution for 15 minutes at room temperature. After washing, phagocytosis was measured with a flow cytometer. Neutrophils were selected through forward and side scatter gating. Phagocytosis is expressed as the mean fluorescence of neutrophils.

Oxidative burst

Freshly isolated human blood was anticoagulated using 50 µg/ml lepirudin. Oxidative burst was initiated upon addition of 100 µl luminol and heat-killed staphylococci (1.5×10^8) to 10% blood in the presence or absence of SSL7 (total volume of 225 µl). The oxidative burst was measured every 30 seconds for 60 minutes at 37°C by chemiluminescence using a luminometer (Berthold Technologies, Bad Wildbad, Germany). Chemiluminescence is expressed as the area under the curve.

Hemolysis assay

In the classical pathway hemolytic assay, NHS and BALB/c mouse serum (NMS, Innovative Research, Novi, MI) were first incubated with sheep or rabbit erythrocytes, respectively, to preclear serum of antibodies directed against erythrocytes. Concurrently, sheep and rabbit erythrocytes were opsonized with anti-erythrocyte IgM. The opsonized rabbit or sheep erythrocytes (2×10^7) were then incubated with 10% precleared NHS or NMS, respectively, in the presence of 0 – 1350 nM SSL7 in HBS²⁺. After 1 hour at 37 °C, the samples were centrifuged, and the absorbance of the supernatants at 405 nm was measured.

Peritoneal Arthus reaction

The Arthus reaction was initiated upon i.v. injection of BALB/c mice with 100 µl of OVA (20 mg/kg of body weight; Sigma-Aldrich), immediately followed by an i.p. injection of 800 µg of rabbit anti-OVA IgG (MP Biomedicals). In inhibition experiments, 60 µg SSL7 was i.v. and i.p. administered 30 minutes prior to initiation of the Arthus reaction. In control experiments, 60 µg SSL7 was administered i.v. and i.p. in the absence of the Arthus reaction. Mice of different treatment groups were killed 6 hours after the onset of the peritoneal Arthus reaction, and the peritoneal cavity was lavaged with 6 ml of ice-cold 0.1% BSA/PBS. Collected peritoneal cells were washed with PBS, and the cell number was adjusted to 5×10^5 cells/ml. 50 µl of this cell suspension was used to prepare cytospin

slides, which were stained with DiffQuick. Neutrophil numbers per square millimeter were calculated from ≥ 20 different microscopic fields. Animal care was provided in accordance with National Institutes of Health guidelines. Animal studies were approved by either the Bezirksregierung Hannover or the Cincinnati Children's Hospital Medical Center institutional animal care and use committee.

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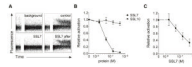


Fig. 1.

SSL7 inhibits generation of C5a during staphylococcal opsonisation. (A,B) Staphylococci were treated with 10% human serum for 30 minutes at 37°C in the presence of 0 – 450 nM SSL7 or SSL10. In one case (“SSL7 after”), 450 nM SSL7 was added to serum supernate after opsonisation. C5a generation was measured by using supernates as stimuli for calcium mobilization in U937-C5aR cells. Results depict in (A) representative calcium mobilization plots and in (B) mean values \pm SEM of three independent experiments and are expressed relative to cells treated with supernate without staphylococcal proteins. In (A), “background” and “control” represent cells stimulated with untreated serum and serum after opsonisation without SSL7, respectively. (C) Cell-bound C5 convertases were constructed on staphylococci by initial deposition of C3b and subsequent addition of soluble fD, fB, and properdin. C5 was subsequently added in the presence of 0 – 450 nM SSL7, and C5a generation in the absence of IgA was analysed by using supernates as stimuli for calcium mobilization in U937-C5aR cells. Results represent mean values \pm SEM of three independent experiments and are expressed relative to cells treated with supernate without SSL7.

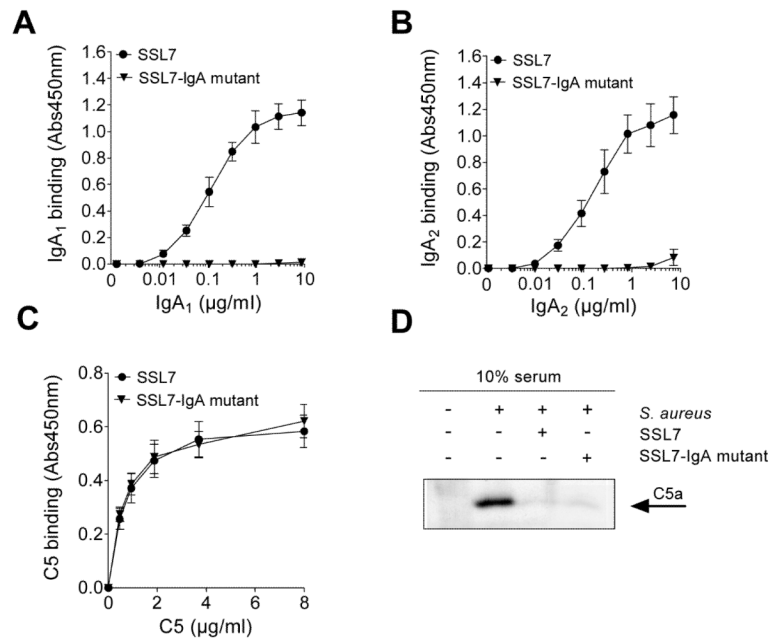


Fig. 2. SSL7 effects are not dependent on IgA binding. (A-C) ELISA experiments showing binding of IgA₁ (A), IgA₂ (B), or C5 (C) to wells coated with 450 nM SSL7 and SSL7-IgA mutant. Results represent mean values \pm SEM of two independent experiments. (D) Staphylococci were opsonized with 10% human serum in the presence of 450 nM SSL7 or SSL7-IgA mutant. Supernatants were examined for generated C5a by Western blotting. Representative of three experiments.

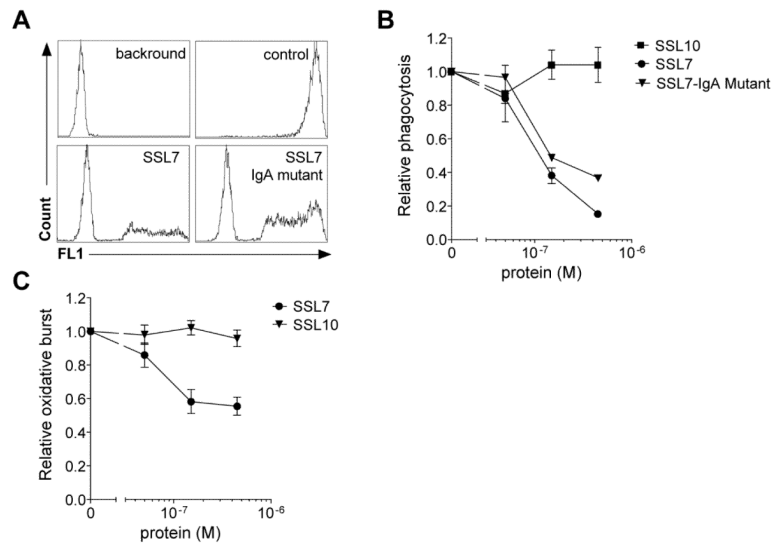


Fig. 3. SSL7 inhibits phagocytosis and generation of oxidative burst in whole human blood. (A,B) Phagocytosis of FITC-labeled staphylococci was measured in whole human blood in the presence of 0 – 450 nM SSL7, SSL7-IgA mutant, or SSL10. (A) Representative histograms depicting background neutrophil fluorescence, neutrophil phagocytosis of bacteria without (control) and with 450 nM SSL7 or SSL7-IgA mutant. (B) Results express phagocytosis of FITC-labeled staphylococci in whole human blood treated with staphylococcal proteins relative to control-treated blood and represent mean values \pm SEM of three independent experiments. (C) After addition of 0 – 450 nM SSL7 or SSL10 and staphylococci to whole human blood, oxidative burst was measured over time. Results are expressed relative to control-treated cells and represent mean values \pm SEM of three independent experiments.

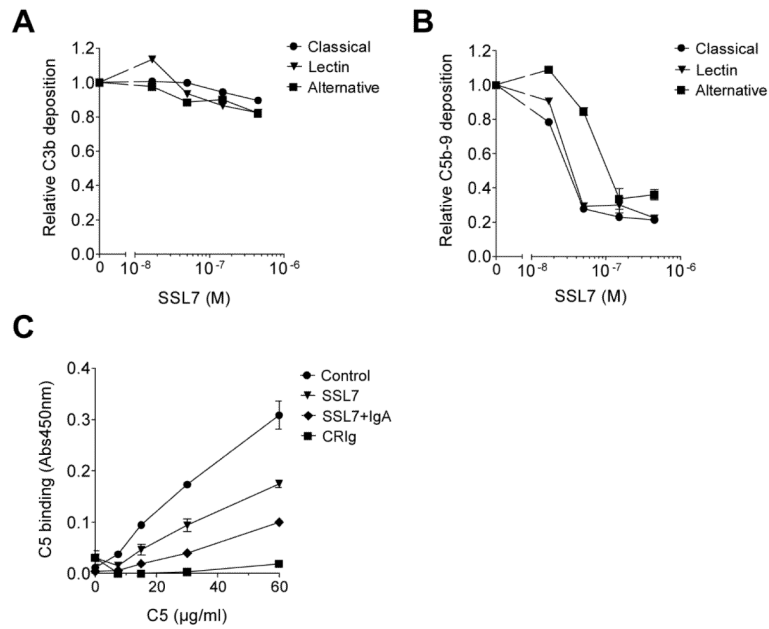


Fig. 4. SSL7 affects binding of C5 to C5 convertases. (A,B) Complement ELISA experiments showing C3b (A) and C5b-9 (B) deposition after complement activation of the classical (5% serum), lectin (5% serum), or alternative (25% serum) pathway in the presence of 0 – 450 nM SSL7. Results are expressed relative to control-treated serum and represent mean values \pm SEM of three independent experiments. (C) Binding of C5 to (C3b)₂ in the presence of 10 μ g/ml CR1g (Complement Receptor of the Immunoglobulin superfamily recognizes C3b molecules), SSL7 (450 nM), or SSL7 (450 nM) and IgA (60 μ g/ml). Results represent mean values \pm SEM of two independent experiments.

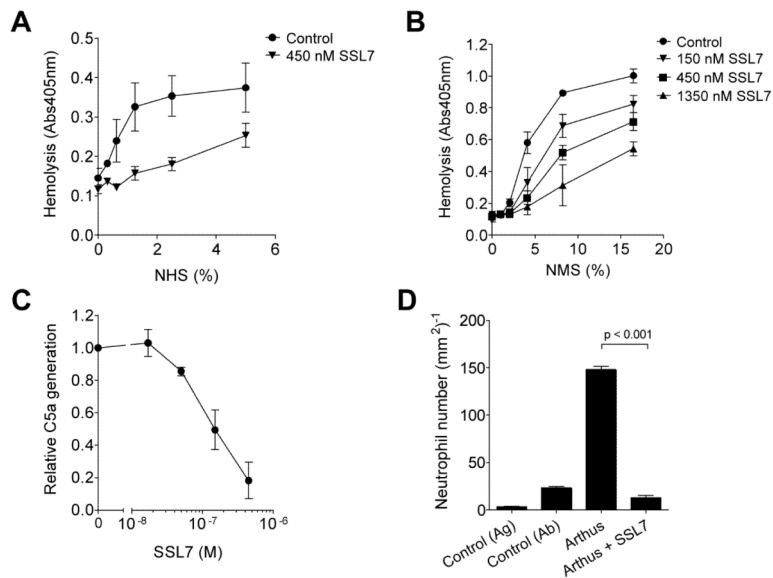


Fig. 5. SSL7 inhibits C5a generation in vivo. (A and B) Classical complement pathway-mediated hemolysis of sheep (A) or rabbit (B) red blood cells in human (A) or mouse (B) serum preincubated with SSL7. Results represent mean values \pm SEM of three independent experiments. NHS and NMS stand for normal human serum and normal mouse serum, respectively. (C) Staphylococci were treated with 30% mouse serum for 30 minutes at 37°C in the presence of 0 – 450 nM SSL7. C5a generation was measured by using supernates as stimuli for calcium mobilization in U937-C5aR cells. Results depict mean values \pm SEM of four independent experiments and are expressed relative to cells treated with supernate without staphylococcal proteins. (D) In the reverse passive Arthus reaction peritonitis model, peritoneal cavity lavage was performed six hours after challenge of mice with immune complexes. SSL7 was injected 30 minutes before challenge with immune complexes or was used as a challenge itself. Collected neutrophils were subsequently quantified. $P < 0.001$ by analysis of variance ($n = 10$ mice per group).