

Structural basis for inhibition of complement C5 by the SSL7 protein from *Staphylococcus aureus*

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***Staphylococcus aureus* secretes the SSL7 protein as part of its immune evasion strategy. The protein binds both complement C5 and IgA, yet it is unclear whether SSL7 cross-links these two proteins and, if so, what purpose this serves the pathogen. We have isolated a stable IgA-SSL7-C5 complex, and our crystal structure of the C5-SSL7 complex confirms that binding to C5 occurs exclusively through the C-terminal β -grasp domain of SSL7 leaving the OB domain free to interact with IgA. SSL7 interacts with C5 >70 Å from the C5a cleavage site without inducing significant conformational changes in C5, and efficient inhibition of convertase cleavage of C5 is shown to be IgA dependent. Inhibition of C5a production and bacteriolysis are all shown to require C5 and IgA binding while inhibition of hemolysis is achieved by the C5 binding SSL7 β -grasp domain alone. These results provide a conceptual and structural basis for the development of a highly specific complement inhibitor preventing only the formation of the lytic membrane attack complex without affecting the important signaling functions of C5a.**

crystallography | innate immunity | structural biology | complement | IgA

The complement system comprises >30 plasma and membrane proteins and is an essential component of innate immunity against Gram-positive bacteria such as *Staphylococcus aureus*. Complement proteins interact directly with surface molecules unique to microorganisms or alternatively to the effector region of bound antibodies. The proteolytic activation of complement results in dramatically enhanced phagocytosis (1), clearance of immune complexes and apoptotic cells (2), and mediation of inflammation (3). Complement activation also promotes adaptive immune responses by serving as a natural adjuvant, enhancing and directing responses by lymphoid cells (4, 5).

Complement activation triggers cleavage of the three homologous 185- to 200-kDa proteins C3, C4, and C5. Three activation pathways converge on C3 cleavage to C3b (6). Activation by the alternative pathway (AP) results from spontaneous hydrolysis of an internal thioester bond in C3 or by deposition of properdin on an appropriate surface (7), whereas the classical pathway (CP) and lectin-mediated pathways are activated by surface-bound immune complexes or mannan binding lectins/ficolins, respectively. Activation of the CP or the lectin pathway generates the surface-bound C3 convertase (a proteolytic enzyme cleaving C3) C4b2a, whereas the AP generates the C3 convertase C3bBb. Both may recruit an additional C3b molecule to form the CP C5 convertase C4b2a3b or the AP C5 convertase C3bBb3b (8, 9), which cleaves C5 to generate the large fragment C5b and the anaphylatoxin C5a. This binds with high affinity to the C5a receptor (CD88) on myeloid cells triggering G protein ($G\alpha_1$ and $G\alpha_{16}$)-mediated cell activation, chemotaxis, respiratory burst, and release of proinflammatory mediators (10). C5b quickly associates with C6, C7, C8, and multiple molecules of C9 to form the membrane attack complex (MAC) that results in rapid cell lysis (11). Elevated levels of C5a are implicated in a wide range of inflammatory disorders, such as rheumatoid arthritis,

ischemia/reperfusion injury, sepsis, and fibrotic conditions (12). MAC deposition through C5b on erythrocytes results in destruction of these cells in the hemolytic disease paroxysmal nocturnal hemoglobinuria (PNH) (13). In addition, excessive MAC formation is linked with the pathophysiology of conditions such as antibody-mediated transplant rejection (14), inflammatory neuropathies (15) and multiple sclerosis (16).

Given its importance to innate immune clearance, pathogens have developed many strategies to prevent complement activation (17). The Staphylococcal Superantigen-Like protein 7 (SSL7) binds to C5 to inhibit complement-mediated hemolytic and bactericidal activity (18). SSL7 is bifunctional and binds to C5 and IgA Fc, thereby blocking both C5 cleavage and recognition of IgA by Fc α RI (18, 19). To decipher the molecular mechanism of complement inhibition by SSL7 and to understand the functional consequences of its dual binding, we have determined the crystal structure of the C5-SSL7 complex at 3.6 Å resolution. We show that SSL7 interacts with both C5 and the cleaved C5b, and that inhibition of C5 cleavage requires the presence of IgA Fc bound simultaneously to SSL7. Our structure allows us to rationalize these results, and a detailed molecular model for the recognition of C5 by the C5 convertase is presented.

Results

Overall Structure. We determined the structure of human C5 in complex with SSL7 cloned from strain 12598 (residues 40–230 traced, 3.6 Å resolution; Table S1 and Figs. S1 and S2) or the C-terminal β -grasp domain of SSL7' (SSL7'c) derived from strain 4227 (residues 129–230 traced, 4.2 Å resolution). Because the protein sequences of SSL7 and SSL7' show variations in the C5 interaction region (Fig. S2), it was of interest to examine complexes with C5 of both versions of SSL7. The structures show that the C-terminal β -grasp domain in SSL7 is almost exclusively responsible for the interaction with C5 (Fig. 1A and B), with the structures of the two complexes being essentially identical except for the absence of the OB domain in the C5-SSL7'c complex. In the C5-SSL7 complex a single electrostatic interaction is formed between E53 in the OB domain and C5 R782* (C5 residue numbers marked by *; prepro numbering used). The binding to C5 has little influence on

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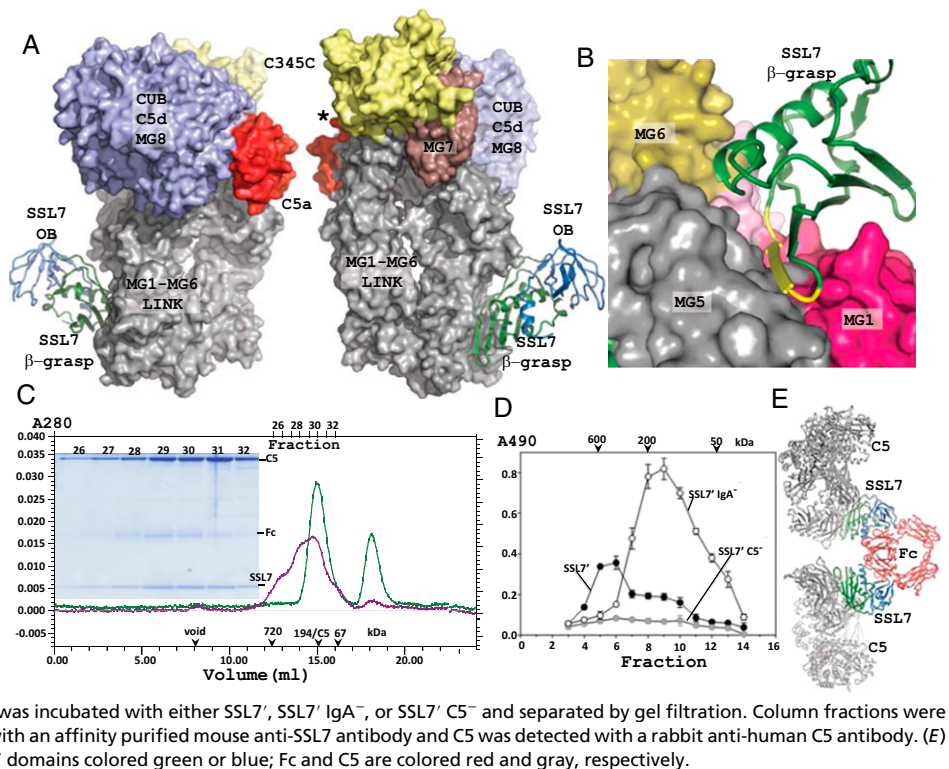
Freely available online through the PNAS open access option.

Data deposition: The coordinates and structure factors for the C5-SSL7 and C5-SSL7'c complexes have been deposited in the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank [IDs 3KLS (C5-SSL7) and 3KM9 (C5-SSL7'c)].

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Fig. 1. The C5-SSL7 complex. (A) Overview of the complex with SSL7 shown in cartoon. The OB domain is colored blue and the β -grasp domain green. C5 is shown in a surface representation. In C5 is the N-terminal MG1-MG6 domains and the linker region are shown in gray. The MG7 domain (brown) links the MG1-MG6 superhelix with the CUB-C5d-MG8 superdomain (blue). The flexibly attached C-terminal C345C domain is colored yellow, and the C5a domain red. In the right panel, rotated around a vertical axis relative to the left panel, the asterisk marks the position of the peptide bond cleaved by the convertase. (B) Close-up on the interaction of the SSL7 β -grasp domain with three MG domains in C5. Residues 144–151 in SSL7 are colored yellow. (C) Superose 6 gel-filtration analysis of the C5+SSL7 (green line) and the C5+SSL7+ IgA Fc complex (magenta line). Considering the elution volume, the Fc-SSL7₂-C5₂ complex is likely to be present together with less saturated complexes. Ticks mark elution point of proteins used for calibration. (C Inset) Nonreducing SDS/PAGE analysis of fractions from gel filtration of C5+SSL7+IgA Fc. The C5+SSL7 experiment was done with excess SSL7, which elute in the peak at 18.3 mL. (D) Formation of SSL7-IgA-C5 complexes in human serum. Human serum was incubated with either SSL7', SSL7' IgA⁻, or SSL7' C5⁻ and separated by gel filtration. Column fractions were tested by sandwich ELISA using plates coated with an affinity purified mouse anti-SSL7 antibody and C5 was detected with a rabbit anti-human C5 antibody. (E) Model of a Fc-SSL7₂-C5₂ complex with the SSL7 domains colored green or blue; Fc and C5 are colored red and gray, respectively.



the structure of SSL7 compared to the structure of the SSL7-IgA Fc complex (19) or the structure of isolated SSL7 (20). A local conformational change occurs in the loop V141-D145 within the stretch E131-L154, which is almost solely responsible for the SSL7 interaction with C5 (Figs. 1B and 2A, and Figs. S1–S3).

Likewise, relative small changes are observed in C5 (Fig. S1B), which at the resolution of 3.6 Å appears to be essentially unchanged relative to the structure of free C5 (21). The interaction of C5 with SSL7 is mediated mainly by the MG1 and MG5 domains with minor contributions from the MG2 and MG6 domains (Figs. 1B and 2A, and Fig. S4). Formation of the C5-SSL7 complex buries 1,919 Å² of surface area, which is even larger than the area of 1,568 Å² buried in the SSL7-IgA Fc interaction. However, the two sets of interactions are of rather different nature. In agreement with our isothermal calorimetry (ITC) measurements (Table S2 and Fig. S5), the C5-SSL7 interface (Fig. 2A and Fig. S3) is dominated by hydrogen bonds and electrostatic interactions, and relatively few hydrophobic interactions (PISA $\Delta^1G = -2.8$ kcal/mol), whereas the SSL7-Fc interface (19) is more hydrophobic (PISA $\Delta^1G = -5.7$ kcal/mol).

IgA-SSL7-C5 Complex. Gel-filtration analysis of combinations of pure C5, SSL7, and IgA Fc (Fig. 1C) show that SSL7 can bind C5 and IgA simultaneously, and strongly suggests that an Fc-SSL7₂-C5₂ pentameric complex (predicted molecular weight, 476 kDa) is formed in solution, along with the partially saturated complexes Fc-SSL7-C5 (263 kDa) and Fc-SSL7₂-C5 (286 kDa). To further examine the relationship between C5 and IgA binding, mutations were introduced to disrupt either or both binding sites. The SSL7' C5⁻ mutant contained a single charge change D147K in the β -grasp domain. The SSL7' IgA⁻ mutant contained three mutations (N68T.L109A.P112A) in the OB-domain that were necessary to significantly deplete IgA binding and the SSL7' C5⁻IgA⁻ mutant combined all four mutations (N68T.L109A.P112A.D147K) (Fig. S6 A and B). The potential of SSL7' to form complexes with IgA and C5 in blood was examined by incubating SSL7', SSL7' IgA⁻, or SSL7' C5⁻ with human serum followed immediately by gel filtration (Fig. 1D). Incubation with

SSL7' resulted in a predominant C5 containing peak eluting at ≈ 600 kDa consistent in size with an IgA-SSL7₂-C5₂ pentameric complex (predicted molecular weight, 590 kDa). Incubation with the SSL7' IgA⁻ mutant produced a single C5 containing peak eluting at ≈ 200 kDa consistent in size with the SSL7'-C5 heterodimer (molecular weight, 213 kDa). The SSL7' C5⁻ mutant formed no detectable C5 containing complexes. The saturated pentameric complex Fc-SSL7₂-C5₂ was modeled (Fig. 1E) by superposition of two copies of the C5-SSL7 complex onto the Fc-(SSL7)₂ structure (19). This model predicts that a pentameric structure is entirely feasible with no clashes between the five molecules. The molar concentrations of IgA and C5 in blood are on the order of 10 and 0.4 μ M, respectively, and so the limiting factor in the formation of this complex would be the concentration of SSL7. Notably, in the Fc-SSL7₂-C5₂ model, neither SSL7 nor IgA Fc are within 70 Å of the cleavage site at R751 in C5, suggesting that IgA forms a scaffold for SSL7 to recruit C5 in such a way as to mask a convertase binding site distant from the C5a domain.

C5-SSL7 Interface. The core of the C5-SSL7 interaction is the anti-parallel pairing of the SSL7 β -strand E144-I150 and the C5 β -strand H511*-E516* with five hydrogen bonds formed between main chain atoms in the two strands (Fig. 2A and Figs. S2–S4). Their pairing leads to formation of a large continuous β -sheet with five strands from the inhibitor and four strands from C5. SSL7 D147 further stabilizes the interaction by electrostatic interactions with C5 R515* and by engaging in a hydrogen bond with the side chain of Y501*. SSL7 is known to strongly bind human, chimp, baboon, pig, sheep, goat, and rabbit C5, but not bovine, rat, or horse C5 (18). Sequence alignment (Fig. S4) suggests that C5 residues 511–516 are important, because this region is strictly conserved in C5 molecules that bind SSL7, whereas there are 1–3 residues with sequence variation in nonbinding C5. Hence, although this stretch forms main chain β -sheet hydrogen bonds with SSL7, even small perturbations to the C5 structure in this area can apparently lead to a significant drop in SSL7 sensitivity.

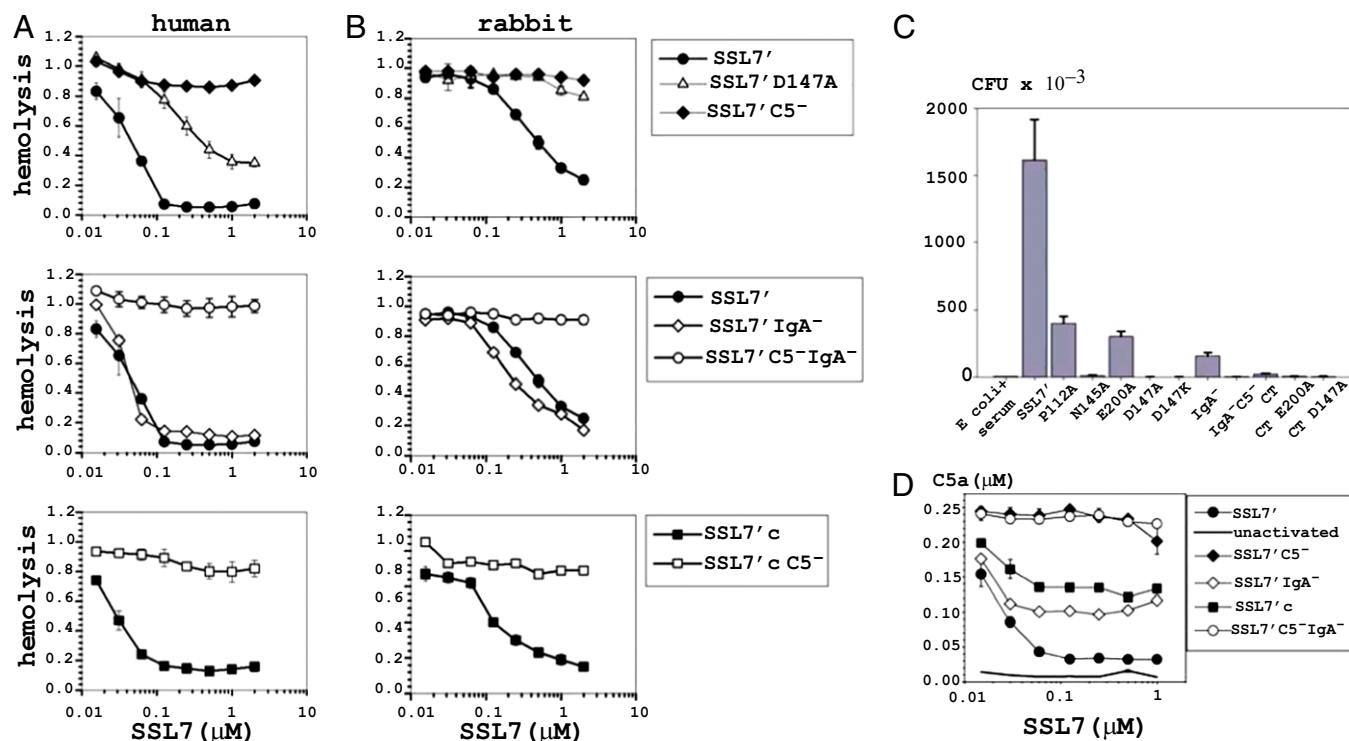


Fig. 3. Consequences of SSL7 mutants defective in C5 and IgA binding on complement C5-mediated functions. (A and B) The ability of SSL7' and SSL7' mutants to inhibit the hemolytic activity in 20% human serum against human red blood cells (A) or 20% rabbit serum against human red blood cells (B). These assays were performed in triplicate and represent the results from a single representative donor. Equivalent results were achieved from multiple donors. (C) Inhibition by 1 μM SSL7' and SSL7' mutants against killing of *E. coli* by 5% human serum. Bacterial survival was enumerated by colony plating in triplicate for each dilution. Results are representative of three repeat experiments. (D) Inhibition by varying concentrations of SSL7' and SSL7' mutants of complement C5a production in response to the addition of 10^7 heat-killed *S. aureus* to 10% cell-free human serum. C5a was quantified by sandwich ELISA, using a commercial C5a as a concentration standard. The results are representative of two separate experiments from a single donor.

needed to efficiently inhibit cleavage of the C5-SSL7 complex. One SSL7 molecule bound directly to C5 does not overlap with C3b in the convertase, whereas IgA Fc bound to this SSL7 overlaps with the C3b at a single loop. A second SSL7 bound to Fc is predicted to significantly constrain the binding of C3b (Fig. 4B and C) and a second C5 molecule bound to the second SSL7 would completely prevent convertase binding to not one but both C5 molecules captured in the complex.

We have noted another potential consequence of SSL7 cross-linking C5 and IgA. Only a single edge of the relatively flat SSL7 molecules appears to be accessible to antibody recognition once the C5-SSL7-IgA complex is formed (Fig. S8). On top of this, the affinity for C5 and IgA is so high that the concentration of neutralizing SSL7 specific IgG will have to be similarly high to achieve neutralization. A similar mechanism for evading aggregation by host antibodies may apply to other pathogen proteins that form large complexes with multiple large abundant host proteins. Several other complement inhibitor proteins from pathogens bind multiple abundant high molecular weight host proteins (24).

SSL7 Binds to C5b. The structure of C5b is unknown but may resemble C3b (25, 26), suggesting that the release of C5a triggers a major conformational change in the C5d-CUB-MG8 superdomain, leaving the MG1-MG6 superhelix largely unchanged (Fig. S9A). Hence, a C5b closely resembling C3b is predicted to bind SSL7. To verify this, we generated C5b with CVF-Bb and then incubated it with His-tagged SSL7. The C5b-SSL7 complex was isolated, and the strength of this intermolecular interaction appeared to be similar to that observed for the C5-SSL7 complex (Fig. S9B). This suggests that there are no substantial changes in

packing of the MG1, MG2, and MG5 domains upon C5 cleavage, because such changes would be expected to affect SSL7 binding.

Discussion

C5 Convertase. There are now two known binding sites for pathogen proteins inhibiting cleavage of C5. OmCI appears to lock the conformation of C5 and in particular the C345C domain (21), whereas SSL7 binds at the opposite end of the molecule between the distal MG1 and MG5 domains. Both inhibitors appear to block C5 cleavage by interfering with convertase recognition far from C5a. Convertase binding seems to require surface regions distributed along the entire length of the C5. In both the AP and CP C5 convertases, an additional molecule of C3b is present in addition to the C3b/C4b and Bb/C2a. This switches the specificity from C3 to C5 by decreasing the K_m for the C5 substrate (27). This additional C3b molecule required to convert the C3 convertase to a C5 convertase is apparently not necessary for the SSL7-IgA-mediated inhibition according to our proteolysis results with the minimal CVF-Bb C5 convertase. Our model of C5 recognition by C3b/C4b is based on an interaction similar to that proposed for C3 with C3b/C4b (23), and the model explains the importance of IgA Fc in SSL7-mediated inhibition of CVF-Bb C5 cleavage. In addition, it agrees with results showing that residues centered around R462 (mature C4 numbering) in the MG5 domain of human C4b are directly involved in C5 recognition by the CP C5 convertase (28).

Inhibition Modes of SSL7. Our model of the IgA-SSL7₂-C5₂ complex (Fig. 1E) does not predict any direct IgA-C5 interactions. But IgA-mediated steric hindrance appears to be the most likely

Crystallization and Structure Determination. Crystals were obtained through vapor diffusion by mixing of 6 mg/mL protein in a 1:1 ratio with the reservoir buffer containing 50 mM MgAc₂ and 50 mM Mes (pH 6.2). Diffraction data were processed with XDS (29) (Table S1). The structure of C5-SSL7 was determined by molecular replacement using PHASER (30). Both models were rebuilt in O (31) and refined with PHENIX (32).

Time Course of C5 Cleavage and C5b-SSL7 Pull-Down. Convertase complexes were formed by mixing C5, Fb, and Fd (Complement Technology) in a molar ratio (relative to 1 mol of C5) of 0.05:0.05:0.0015 in 20 mM Tris/150 mM NaCl/10 mM MgCl₂ (pH 8.0) at 37 °C for 1 h. Before cleavage, C5 alone or mixed with a 4-fold molar excess of SSL7 or SSL7 + IgA-Fc was incubated at 37 °C for 1 h before mixing with the preformed convertases and digestion took place at 37 °C for up to 4 h. SSL7 pull-down experiments with C5 or C5b were done by incubating Ni²⁺-NTA agarose with either C5-SSL7 or C5b-SSL7. The intensities of the α - and α' -chains in C5/C5b were quantified with ImageJ.

Biophysical Measurements. The ITC experiments were performed at 30 °C in a VP-ITC instrument from MicroCal and data were analyzed using the Origin software package.

Inhibition of sMAC (sC5b-9) Formation. SSL7 and SSL7 mutants (20 μ M in phosphate-buffered saline) were diluted to 0.4 μ M in normal human serum and incubated at 37 °C for 4 h. Sera with addition of buffer only incubated at 37 °C or 4 °C (no activation) were used as controls. The generation of sMAC was determined by an immunofluorometric assay (33), and the data in Fig. 2B represent the mean and standard deviation for six experiments with each SSL7 variant.

Serum Hemolytic Assay. RBCs from human or sheep (Invitrogen; Alsevers sheep blood) were combined with human and rabbit serum, respectively. The RBCs were standardized to 2×10^8 cells per mL. SSL7 protein was added to 96-well U-bottom tissue culture plates (Falcon) to give a 2-fold dilution series and added 100 μ L of diluted serum diluted with GHB (150 mM NaCl, 5 mM hepes, 0.11 (wt/vol) Gelatin from bovine skin type B)/MgEGTA. Fifty micro-

liters of RBCs was added, and the plate was incubated for 1 h at 37 °C with periodic shaking. The cells were pelleted by centrifugation, 100 μ L of the supernatant was added to 150 μ L of ice-cold 150 mM NaCl in 96-well, flat-bottom tissue culture plates (Falcon), and the absorbance at 412 nm was measured using a uQuant plate reader (BioTek).

Serum Cell-Free Bactericidal Assay. Sera were allowed to coagulate, and serum was centrifuged for $5,000 \times g$ for 5 min before use. Cell-free serum bactericidal activity was assessed using a fresh overnight culture of *E. coli* K12 strain DH5 α . SSL7 protein was preincubated with 5% normal human serum diluted in Hanks's buffered saline solution (HBSS) (Sigma Aldrich) for 30 min at 37 °C in borosilicate glass tubes and then incubated with $\sim 1 \times 10^7$ stationary-phase ($A_{600} \sim 0.15$) DH5 α cells for 90 min at 37 °C. Tubes were placed in ice to stop reactions, and then a dilution series of each was prepared in HBSS and then plated in triplicate on LB agar and then incubated overnight at 37 °C. Colony-forming units (CFU) were enumerated the following day on a BacCount (BioTek) and expressed as mean CFU \pm SD.

Detection of Serum C5a Production. One hundred-microliter samples of 25% serum were mixed with 50 μ L of serially diluted SSL7 or SSL7 mutant proteins before the addition of 100 μ L of PBS-BSA containing 10^7 heat-killed *S. aureus*. Plates were incubated at 37 °C for 30 min and then centrifuged for 5 min at $1,250 \times g$ to pellet bacteria, and the amount of C5a formed was quantified with a C5a mAb-based sandwich ELISA.

Further details of experimental procedures are described in the *SI Materials and Methods*.

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