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Staphylococcal Complement Inhibitor Modulates Phagocyte Responses by Dimerization of Convertases

Ilse Jongerius,* Manon Puister,* Jin Wu,* Maartje Ruyken,* Jos A.G. van Strijp,* and Suzan H.M. Rooijakkers*

The human pathogen Staphylococcus aureus produces several complement-evasion molecules that enable the bacterium to withstand the host immune response. The human-specific staphylococcal complement inhibitor (SCIN) blocks the central C3 convertase enzymes that trigger critical complement functions, such as C3b deposition, phagocytosis, and C5a generation. SCIN effectively blocks the conversion of C3 by alternative pathway C3 convertases (C3bBb), but also induces dimerization of these enzymes. In this study, we show that 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. These studies provide critical insights into the unique immune-evasion strategies used by S. aureus.

The human complement system is important to combat invading microorganisms. Its activation leads to coverage of target surfaces with C3b, resulting in phagocytosis and the formation of C5 convertases that generate C5a, which is important for the chemotaxis of neutrophils toward the site of infection (1–3). The complement system comprises three pathways: the classical pathway (CP), the lectin pathway (LP), and the alternative pathway (AP). Activation of these pathways results in the formation of C3 convertases, C4b2a (CP/LP) and C3bBb (AP), which cleave C3 (4–6). Cleavage of C3 into C3b leads to remarkable conformational changes in the C3b molecule, allowing covalent binding of its thioester-containing domain to the target surface and exposure of essential binding sites for complement receptors (CRs) on phagocytic cells and complement regulators (7, 8). C3b also supports the formation of C5 convertases, C4b2a3b (CP/LP) and C3bBb (AP), which cleave C5 to generate the C5b-9 membrane attack complex (9, 10).

Phagocytosis of microbes is an essential host defense to clear pathogens. Phagocytic cells, including neutrophils, macrophages, and Kupffer cells, express several CRs that recognize bacterium-bound C3b or iC3b. The formation of iC3b occurs after the cleavage of deposited C3b by factor I (fI) together with cofactors factor H (fH) or CR1. Neutrophils express CR1 and CR3, whereas macrophages express CR3 and CR4. The CR of the Ig superfamily (CR1g) is exclusively found on liver Kupffer cells (11–13). All CRs recognize C3b or iC3b, although through distinct binding sites (8, 14–16).

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

Materials and Methods

Protein expression and purification

C3 was purified from freshly isolated human plasma, and C3b was generated as described previously (21). Preparation of rSCIN was described previously (20). In the SCIN mutant that forms monomeric SCIN-convertases (SCINΔC3b2), residues 5-8-61-64-67-68 were exchanged for alanines. Factor B (fB) was purified from human plasma as described previously (21), and factor D (fD), fH, fI, and iC3b were obtained commercially (Calbiochem, San Diego, CA). rCR1 was a kind gift from Prof. John Atkinson (Washington University, St. Louis, MO); rCR1g-S, the short form of CR1g that binds C3b and iC3b, was obtained from Genentech (South San Francisco, CA).

Cell lines and maintenance

A CHO cell line expressing CR1 (CR1-CHO) and a control R-CHO cell line were obtained from Prof. John Atkinson (22). Cells were maintained in Ham’s F12 medium (Life Technologies, Carlsbad, CA) with 10% FCS.
10 μg/ml gentamicin, and 500 μg/ml neomycin (Life Technologies). A Jurkat cell line expressing CR1g (Jurkat-huCR1g) and Jurkat cells (negative control) (Genentech) (12) were maintained in RPMI 1640 medium containing 10% FCS, 10 μg/ml gentamicin, and 500 μg/ml neomycin.

**Convertase inhibition assay**

C3 (250 nM), C3b (50 nM), fB (500 nM), fD (500 nM), and SCIN or SCIN\textsubscript{C3b2} (2 μM) were incubated in HBS-Mg (HEPES-buffered saline, 20 mM HEPES, 140 mM NaCl, 2.5 mM MgCl\textsubscript{2}, pH 7.4) for 10 min at room temperature, and C3 conversion was analyzed by SDS-PAGE under reducing conditions (21).

**Convertase binding assays**

Fluid-phase SCIN-convertases were prepared as described previously (21). Briefly, 100 μg/ml C3b, 50 μg/ml fB, 5 μg/ml fD, and 10 μg/ml his-SCIN or his-SCIN\textsubscript{C3b2} were incubated in HBS-Mg for 1 h at 4˚C, after which the C3-convertases were pulled down by incubation with magnetic cobalt beads (Dynabeads Talon, Invitrogen, Breda, The Netherlands) for 1 h at 4˚C. Beads were washed three times with cold PBS, 0.05% TWEEN, and complexes were eluted with PBS containing 50 mM EDTA. The amount of C3b present in SCIN-convertases was analyzed using SDS-PAGE. Results were calculated when the amount of C3b molecules in the SCIN- or SCIN\textsubscript{C3b2}-convertases were different from the amount of soluble C3b.

**Complement deposition and CR1 binding to the bacterial surface**

C3b deposition was performed by incubation of *S. aureus* strain Wood 46 (5 × 10\textsuperscript{6} CFU/ml) with 10% human serum in the presence or absence of 10 μg/ml SCIN or SCIN\textsubscript{C3b2} for 30 min at 37˚C. Bacteria were washed, dissolved in PBS, and left at 37˚C for several decay periods. After decay, bacteria were washed again and boiled in sample buffer. Bb formation was analyzed by Western blotting using anti-IgG Abs (Quidel, San Diego, CA). For analysis of the fluorescence intensity of 5000 cells, and the mean fluorescence (FL) was calculated using BD CellQuest Pro software (BD Biosciences, Franklin Lakes, NJ).

**Convertase stabilization assays**

Convertase stability on bacterial surfaces was analyzed by incubation of *S. aureus* strain Wood 46 (5 × 10\textsuperscript{6} CFU/ml) with 10% human serum in the presence or absence of 10 μg/ml SCIN or SCIN\textsubscript{C3b2} for 30 min at 37˚C. Bacteria were washed, dissolved in PBS, and left at 37˚C for several decay periods. After decay, bacteria were washed again and boiled in sample buffer. Bb formation was analyzed by Western blotting using anti-IgG Abs (Quidel, San Diego, CA). For analysis of the fluorescence intensity of 5000 cells, and the mean fluorescence (FL) was calculated using BD CellQuest Pro software (BD Biosciences, Franklin Lakes, NJ).

**C3b cleavage by fH and fI**

Purified C3b (100 μg/ml) and SCIN- and SCIN\textsubscript{C3b2}-convertases were incubated with 15 μg/ml fH and 5 μg/ml fI for 2 h at room temperature. C3b conversion into iC3b was analyzed by SDS-PAGE under reducing conditions and silver staining.

**Phagocytosis**

Phagocytosis was performed as described previously (20). Briefly, FITC-labeled *S. aureus* strain KY27 was incubated with human serum for 2 min in the presence or absence of 10 μg/ml SCIN or SCIN\textsubscript{C3b2} at 37˚C, after which freshly isolated neutrophils were added for 15 min. Phagocytosis was analyzed by flow cytometry. Cells were gated on the basis of forward and side scatter properties. Fluorescence intensity of 10,000 gated neutrophils was determined, and the mean FL was calculated using BD CellQuest Pro software.

**Results**

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

The structure of C3bBb in complex with SCIN revealed that SCIN has a dimerization site that enables the generation of dimeric (C3bBb(SCIN)\textsubscript{2}) instead of monomeric (C3bBbSCIN) convertases (21). To study the role of convertase dimerization by SCIN, we constructed a SCIN mutant (SCIN\textsubscript{C3b2}) that only forms monomeric convertases by replacing all residues involved in dimerization into alanines (Fig. 1A). This is a more precise mutant than the previously described SCIN mutant ChC3b2, in which we exchanged residues 1–13, 59–61, 64, 65, 67, and 68 with corresponding residues from open reading frame D, not only mutating the binding residues but other residues as well (21). SCIN\textsubscript{C3b2} forms stable monomeric convertase enzymes (Fig. 1B) that have the same half-life as dimeric SCIN convertases (21) (4 h at 20˚C; data not shown). To investigate whether dimerization of AP convertases by SCIN is necessary for the inhibition of C3 conversion by convertases in fluid phase, we incubated C3 with C3b, fH, and fD in the presence or absence of SCIN or SCIN\textsubscript{C3b2}.

![FIGURE 1.](http://www.jimmunol.org/) A. Convertase dimerization by SCIN is not necessary for convertase inhibition. Representation of the active C3 convertase (C3bBb; left) and the inhibited convertase in the presence of SCIN (dimeric SCIN-convertase; middle) or the SCIN dimerization mutant (monomeric SCIN-convertase; right). B, Native gel electrophoresis of AP C3 convertases formed in the presence of wild-type SCIN or SCIN\textsubscript{C3b2}, C3b, fH, and fD were incubated with his-SCIN or his-SCIN\textsubscript{C3b2} and purified by cobalt beads. C, C3 conversion by fluid-phase C3 convertases. C3 was incubated with C3b, fH, and fD in the presence or absence of SCIN or SCIN\textsubscript{C3b2}, and C3 conversion was analyzed by SDS-PAGE under reducing conditions. B and C are representative figures of three separate experiments.
shows that SCINΔC3b₂, which forms monomeric convertases, inhibits C3 conversion equally well as wild-type SCIN, which induces dimeric convertases.

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

Although wild-type SCIN and SCINΔC3b₂ inhibit C3 conversion equally in fluid-phase conditions, this may be different on the bacterial surface. Therefore, we analyzed phagocytosis of *S. aureus* by incubating the bacteria with serum and freshly isolated neutrophils in the presence of SCIN or SCINΔC3b₂. We observed that SCINΔC3b₂ had a markedly reduced ability to block phagocytosis compared with SCIN. Wild-type SCIN completely inhibited phagocytosis, whereas SCINΔC3b₂ only inhibited it by <50% (Fig. 2A).

Because the phagocytic uptake of bacteria by neutrophils is dependent on C3b and iC3b, the deposition of these molecules on the bacterial surface was studied. Identical to our findings for fluid-phase C3 conversion, no difference in C3b deposition on the bacterial surface was found between SCIN and SCINΔC3b₂ (Fig. 2B). In addition to that, we observed that iC3b was not formed on the bacterial surface in the presence of SCIN or SCINΔC3b₂ (Fig. 2C), suggesting that SCIN protects C3b from degradation by fH and fI. We prepared soluble SCIN-convertases by incubating C3b, fB, fD, and his-SCIN or his-SCINΔC3b₂ and purified them using cobalt beads. The amount of C3b molecules present inside the complexes was determined via SDS-PAGE (Supplemental Fig. 1). Subsequently, the generated fluid-phase convertases were incubated with fH and fI, after which iC3b formation was analyzed. Fig. 2D demonstrates that soluble C3b is cleaved by fH and fI into iC3b, as revealed by the disappearance of the C3b α-chain, whereas C3b molecules incorporated in the dimeric or monomeric SCIN-convertases are not. Thus, dimerization of convertases by SCIN is important for phagocytosis inhibition; however, this cannot be attributed to differences in C3b deposition or iC3b formation.

**SCIN-induced dimerization of convertases blocks recognition of C3b by CRs**

As demonstrated above, differences in phagocytosis inhibition between SCIN and SCINΔC3b₂ cannot be explained by differences in C3b and iC3b deposition. To investigate whether the recognition of C3b by CRs is impaired by SCIN, we first compared the binding of soluble dimeric and monomeric SCIN-convertases to purified CR1 and CR1g by using an ELISA. Dimeric SCIN-convertases showed a lower binding to CR1 and CR1g than the monomeric convertases or soluble C3b (Fig. 3A). We did not observe a difference in the binding of dimeric and monomeric complexes to an anti-C3 Ab, indicating that equal amounts of C3b molecules were present inside the SCIN-convertases (Fig. 3A).

Next, we studied the binding of soluble SCIN-convertases to surface-expressed CRs. Binding of soluble C3b and SCIN dimeric or monomeric convertases to cells expressing CR1 and CR1g also showed that the binding of dimeric SCIN-convertases to CR1 and CR1g was reduced compared with soluble C3b and the monomeric convertases (Fig. 3B, 3C). Also, the binding of dimeric SCIN-convertases to human neutrophils was reduced compared with C3b and monomeric convertases (Fig. 3D).

In conclusion, dimerization of convertases by SCIN leads to impaired binding of C3b to purified and cell-bound CRs, explaining the difference between SCIN and SCINΔC3b₂ in phagocytosis inhibition.

**Convertase dimerization occurs on bacterial surfaces and enhances convertase stability**

CR1 and CR1g bind less well to soluble dimeric SCIN-convertases than to monomeric convertases. To study whether dimerization of convertases by SCIN occurs on the bacterial surface, bacteria were incubated with human serum in the presence or absence of SCIN or SCINΔC3b₂, and C3b deposition or CR1 recognition was

![FIGURE 2](http://www.jimmunol.org/) Convertase dimerization by SCIN is important for phagocytosis inhibition. A. Phagocytosis of *S. aureus* strain KV27 by freshly isolated neutrophils in the presence of normal human serum and 10 µg/ml SCIN or SCINΔC3b₂. B. C3b deposition via the AP on *S. aureus* in the presence of SCIN or SCINΔC3b₂ (both at 10 µg/ml). C. C3b formation on the bacterial surface in the presence of SCIN or SCINΔC3b₂ (both at 0, 3, or 10 µg/ml) or in F-deficient serum (ΔfH). D. C3b formation in fluid phase analyzed by SDS-PAGE under reducing conditions. C3b (100 µg/ml) was incubated with fH (15 µg/ml) and fI (5 µg/ml) at room temperature, and iC3b formation was measured after 2 h (T = 2); T = 0 shows the amount of C3b molecules at time point zero. A and B represent the mean ± SEM of three separate experiments, whereas C and D are representatives of three separate experiments. See Supplemental Fig. 2 for representative histograms.
measured. SCIN and SCINΔC3b2 equally inhibited C3b deposition (data not shown), but the binding of purified CR1 to bacteria opsonized in the presence of SCIN was less than the binding to bacteria opsonized in the presence of SCINΔC3b2 (Fig. 4A). This indicates that dimeric convertases are formed on the bacterial surface, hiding bacteria from CR1 recognition.

We previously proposed that the dimeric nature of SCIN-convertases could also contribute to the stability of the surface-bound complex (21). Although we demonstrated in this study that the stabilization of dimeric versus monomeric convertases during fluid-phase conditions does not differ, we investigated stabilization properties on the bacterial surface as well. Bacteria were first incubated with human serum, after which Bb stabilization was measured. Fig. 4B and 4C show that SCIN stabilizes convertases with a half-life of 4 h at 37˚C on the bacterial surface compared with a half-life of 2 h for the monomeric variant.

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

Discussion

*S. aureus* secretes several complement-evasion molecules that enable bacterial survival in the human host. These molecules have unique functional properties and block the complement cascade at different steps (18, 26). For instance, staphylokinase activates human plasminogen at the bacterial surface, thereby removing C3b, which results in the inhibition of phagocytosis (27). The secreted proteins extracellular fibrinogen binding protein and extracellular complement binding protein target C3b-containing convertases, thereby inhibiting C3b deposition via the AP and C5a generation in vitro and in vivo (28). In addition, staphylococcal superantigen-like 7 directly binds C5 (29), which leads to the inhibition of C5a formation and neutrophil migration in vitro and in vivo (J. Bestebroer, P.C. Aerts, S.H.M. Rooijakkers, M.K. Pandey, J. Kohl, J.A.G. van Strijp, and C.J.C. de Haas, unpublished observations). SCIN inhibits C3 convertases of the CP, LP, and AP, which affects the complement system at a very early stage; this inhibits C3b deposition as well as phagocytosis and C5a generation. The interaction between SCIN and C3 convertases was shown to be highly human specific, which limits studies in animal models. Nevertheless, SCIN is found in 90% of clinical *S. aureus* strains and is expressed in vivo (20). This, and its location on a human-specific bacteriophage (30), implicates a critical role for SCIN in the pathogenesis of *S. aureus* infections in humans.

The crystal structure of the AP C3 convertase in complex with SCIN revealed a convertase dimerization site in SCIN that seemed unimportant for blocking convertase activity in fluid phase (21). It was unclear whether dimerization of AP convertases by SCIN was a fluid-phase phenomenon. This paper shows that convertase dimerization results in impaired recognition of C3b by CR1 and CRIg. A. Binding of soluble C3b or SCIN-convertases to microtiter plates coated with anti-C3c, CR1, or CRIg. Data represent the mean ± SEM of three separate experiments; ** p < 0.01. Binding of soluble C3b and dimeric or monomeric SCIN-convertases to cell lines expressing CR1 (B) and CRIg (C) or freshly isolated human neutrophils (D). Data represent the mean ± SEM of three separate experiments. See Supplemental Fig. 2 for representative histogram.
dimerization also occurs on bacterial surfaces and that it is critical for the immune-evasion properties of SCIN. We observed major differences in the antiphagocytic activity of SCIN and its dimerization mutant. Fig. 5A represents a surface representation of the C3b molecule with highlighted CR binding sites (8, 32, 33). A recent costructure of CRIg and C3b revealed that CRIg binds the back of C3b (16). Because this site is close to the C3b–C3b interface found in the dimeric SCIN-convertases, it is likely that steric hindrance prevents CRIg from binding C3b within the dimerized, but not the monomeric, complex (Fig. 5B) (21). Binding sites for CR1 are not fully mapped, and future studies are needed to understand the molecular basis for impaired CR1 binding to the convertase dimer. Recent surface plasmon resonance studies showed that SCIN impairs decay acceleration of the convertase by fH (31). We also found that the fH- and fI-mediated conversion of C3b to iC3b is blocked in SCIN-inhibited convertases (monomeric and dimeric). In this way, SCIN prevents formation of iC3b on the surface and indirectly provides a way to evade CR3 recognition as well. Dimeric convertases on the surface are more stable, which help the bacteria to evade the complement system for a long period of time. Because CRIg on liver Kupffer cells is critical for the clearance of complement-coated pathogens from the blood, this prolonged stability of dimeric convertases also enables S. aureus to escape host defenses in later infection stages, when bacteria have disseminated from specific tissue sites to the blood (12). Although it is known that immune-evasion molecules excreted by S. aureus can interfere with recognition by CR2 on B cells (34, 35), SCIN is the first staphylococcal molecule that specifically prevents bacterial recognition by CRs on phagocytic cells.

SCIN blocks the recognition of deposited C3b by CRs. A combination of these events is probably needed to ensure effective protection from the host immune response. The use of SCIN as a therapeutic in inflammatory diseases is limited by the presence of preexisting Abs in humans (19). However, studying the way that bacteria counteract the immune response will be critical in the development of novel antiinflammatory compounds. Our recent costructure and the currently presented insights support the development of SCIN derivatives or analogs that effectively modulate complement activation and phagocytosis.

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