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

*J Immunol* published online 30 November 2009
http://www.jimmunol.org/content/early/2009/11/30/jimmunol.0902865

---

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2009/12/08/jimmunol.0902865.DC1

**Subscription**

Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
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 Journal of Immunology, 2010, 184: 000–000.

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 C3 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, C4b2aC3b (CP/LP) and C3bBb (AP), which cleave C5 to generate the chemoattractants C5a and C5b that are important for the formation of the 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 (CRIg) 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 phagocyte 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 SCIN was described previously (20). In the SCIN mutant that forms monomeric SCIN-convertases (SCIN<i>C3b</i>2), 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, and iC3b were obtained commercially (Calbiochem, San Diego, CA). rCR1 was a kind gift from Prof. John Atkinson (Washington University, St. Louis, MO); rCRlg-S, the short form of CRlg 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 SCINAc3b2 (2 μM) were incubated in HBS-Mg (HEPES-buffered saline, 20 mM HEPES, 140 mM NaCl, 2.5 mM MgCl2, 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-SCINAc3b2 were incubated in HBS-Mg for 1 h at 4˚C, after which the SCIN-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 excluded when the amount of C3b molecules in the SCIN- or SCINAc3b2-convertases were different from the amount of soluble C3b.

Microtiter plates (Greiner Bio One, Alphen a/d Rijn, The Netherlands) were coated overnight with 5 μg/ml anti-C3s WM1 [American Type Culture Collection, Manassas, VA (23)], rCR1, or rCRIg-S. Plates were blocked with PBS, 0.1% Tween, and 4% BSA for 1 h at 37˚C. Plates were washed three times between every step with PBS with 0.1% Tween. Soluble C3b (60 μg/ml) and fluid-phase SCIN- and SCINAc3b2-convertases were added for 1 h at 4˚C, after which binding was detected using peroxidase-labeled anti-C3 Abs (1:5000) (Protos Immunoresearch, San Francisco, CA). Data were analyzed by a two-tailed unpaired Student t test. The binding of soluble C3b or SCIN-convertases to cells expressing CR1 or CR1g was studied by the incubation of various concentrations of fluid-phase convertases or 60 μg/ml SCIN or SCINAc3b2 to cells expressing CR1 or CRIg, 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 (C3bBhSCIN)2 instead of monomeric (C3bBhSCIN) convertases (21). To study the role of convertase dimerization by SCIN, we constructed a SCIN mutant (SCINAc3b2) 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). SCINAc3b2 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, fB, and fD in the presence or absence of SCIN or SCINAc3b2. Fig. 1C shows a representative 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 SCINΔC3b3 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...
SCIN inhibits phagocytosis by C3bBb-dimerization

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.

**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.
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 sterial 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.

**Acknowledgments**

We thank John Atkinson and Kathy Liszewski for providing rCR1 and CR1-CHO cells, Menno van Lookeren-Campagne and Genentech for providing CRIg-S and Jurkat-CRIg cells.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


Supplementary figure 1: Quantification of C3b molecules in purified SCIN-convertases

The concentration of C3b molecules present in purified SCIN- or SCINΔC3b2-convertases was analyzed by SDS-PAGE and silverstaining. Representative for all experiments performed with purified convertases.

Supplementary figure 2: Flow cytometry histograms.

Representative histograms of flow cytometry data obtained for C3b deposition, phagocytosis and C3b binding to CRO-CR1 cells (similar to histograms obtained for PMN and Jurkat-CRIg cells).