Production of Staphylococcal Complement Inhibitor (SCIN) and Other Immune Modulators during the Early Stages of *Staphylococcus aureus* Biofilm Formation in a Mammalian Cell Culture Medium

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ABSTRACT Immune modulators are known to be produced by matured biofilms and during different stages of planktonic growth of *Staphylococcus aureus*. Little is known about immune modulator production during the early stages of biofilm formation, thus raising the following question: how does *S. aureus* protect itself from the innate immune responses at these stages? Therefore, we determined the production of the following immune modulators: chemotaxis inhibitory protein of staphylococci (CHIPS); staphylococcal complement inhibitor (SCIN); formyl peptide receptor-like 1 inhibitor; gamma-hemolysin component B; leukocidins D, E, and S; staphylococcal superantigen-like proteins 1, 3, 5, and 9; and staphylococcal enterotoxin A. Production was determined during *in vitro* biofilm formation in Iscove's modified Dulbecco's medium at different time points using a competitive Luminex assay and mass spectrometry. Both methods demonstrated the production of the immune modulators SCIN and CHIPS during the early stages of biofilm formation. The green fluorescence protein promoter fusion technology confirmed *scn* (SCIN) and, to a lesser extent, *chp* (CHIPS) transcription during the early stages of biofilm formation. Furthermore, we found that SCIN could inhibit human complement activation induced by early biofilms, indicating that *S. aureus* is able to modulate the innate immune system already during the early stages of biofilm formation *in vitro*. These results form a stepping stone toward elucidating the role of immune modulators in the establishment of biofilms *in vivo* and present opportunities to develop preventive strategies.

KEYWORDS early-stage biofilm, IMDM, immune modulators, SCIN

Infections with *Staphylococcus aureus*, an opportunistic pathogen, have been reported in more than 50% of soft tissue infection and osteomyelitis cases (1–7). Chronic infections, such as osteomyelitis, are strongly associated with the formation of biofilms (7–10), which are characterized by clustered bacteria with heterogeneous metabolic activity (11–16). These clustered bacteria are embedded in an extracellular matrix composed of extracellular DNA (eDNA), proteins, and polysaccharides from endogenous and exogenous origins (11, 17–21). *S. aureus* biofilm-related infections are of concern as they are associated with treatment failure and can act as persistent source foci of infections (17, 22, 23). The poor treatability of these staphylococcal biofilm-associated infections is ascribed to the poor penetration of host immune responses and antibiotics into biofilms (23, 24), together with the presence of persister bacteria (12, 17, 25).

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Generally, *S. aureus* biofilm formation has four stages (26, 27): attachment, adhesion-multiplication, maturation, and dispersion. Attachment, occurring after 1 to 2 h of growth, involves elevated expression of cell wall-associated adhesins (CWA) (15, 21, 28, 29). In the adhesion-multiplication stage, bacteria multiply and form small aggregates known as microcolonies; aggregate formation is also mediated by CWA proteins, including fibrinogen- and fibronectin-binding proteins (11, 28, 30). Moormeier et al. (31) have proposed an additional stage, exodus, following the multiplication stage. During this stage, DNase production facilitates the release of a subset of adhered bacteria and, thus, the formation of tower-like structures (31). Next is the maturation phase, in which multilayered cell clusters form within a matrix of eDNA, proteins, and polysaccharides (18–20, 32–35). During this stage, several molecules that are described to have immune-modulating activity are produced (11, 36–41). The final stage of biofilm formation is dispersion, where the bacterial cells begin to dissociate from the biofilm as the result of protease and DNase production, promoting bacterial detachment and seeding (14, 27, 42, 43).

Mature biofilms of *S. aureus* are known to produce immune modulators (11, 14, 37); thus, the following questions are raised: what about the early stages of biofilm formation, and can *S. aureus* escape from the quick response of the complement system and opsonophagocytosis by neutrophils during this stages as well? In a previous study, it was shown that chemotaxis inhibitory protein of staphylococci (CHIPS) and staphylococcal complement inhibitor (SCIN) can be produced during the early stages of planktonic growth of *S. aureus* and can inhibit chemotaxis and, subsequently, phagocytosis by host immune cells (44). This in combination with the findings of earlier work from our group, in which we observed an increased IgG4 response toward CHIPS, SCIN, formyl peptide receptor-like 1 inhibitor (FLIPr), gamma-hemolysin component B (HlgB), leukocidin D (LukD), LukE, LukS, staphylococcal superantigen-like protein 1 (SSL1), SSL3, SSL5, SSL9, and staphylococcal enterotoxin A (SEA) in the serum of healthy controls and patients suffering from various *S. aureus*-related infections (45), which made us hypothesize that these virulence factors might be produced by biofilm-associated bacteria. The pathogenic mechanisms of these proteins are described in Table 1. We therefore initially studied the production of these 12 virulence factors during the different stages of biofilm formation of *S. aureus* in Iscove's modified Dulbecco's medium (IMDM), a mammalian tissue culture medium, using a competitive Luminex assay and confirmed the data by mass spectrometry. The transcription of *chp* (the gene encoding CHIPS) and *scn* (the gene encoding SCIN) was studied by use of the green fluorescence protein (GFP) promoter fusion technology. Lastly, we ascertained the effect of SCIN on complement activation during the early stages of biofilm formation.

RESULTS

Biofilm formation. Biofilm formation is one of the important virulence factors of *S. aureus*. The ability to form a biofilm in mammalian cell culture medium was assayed by incubating our clinical isolates M82 (sequence type 20 [ST20]), M116 (ST239), and RWW50 (USA300) and the laboratory strains Newman and RN6390 in a polystyrene microplate containing IMDM at 37°C. All tested strains produced detectable biofilms after 2 h, and the biofilm mass increased in a time-dependent manner (Fig. 1A). Biofilm formation in the first hour of formation could not be visualized with the crystal violet staining procedure. This is most likely a consequence of either a very low density of bacteria or the fact that the bacteria were not yet firmly attached to the polystyrene surface and were thus washed away during the staining procedure.

Detection of immune modulator production of *S. aureus* biofilms by competitive Luminex assay. Using the competitive Luminex assay (46, 47), we monitored the production of the immune modulators CHIPS, SCIN, FLIPr, HlgB, LukD, LukE, LukS, SSL1, SSL3, SSL5, SSL9, and SEA (Table 1) during the 2nd, 4th, and 24th hours of biofilm formation. The assay detected SCIN in the supernatants of biofilms during the 4th hour of biofilm formation of the M82, M116, USA300, and Newman strains (Fig. 1C to F). The amount of SCIN present in the 4th-hour supernatants of Newman biofilms was signif-

TABLE 1 Immunomodulation mechanism of selected *S. aureus* proteins

<i>S. aureus</i> protein	Pathogenic mechanism ^a	Reference(s)
CHIPS	CHIPS inhibits phagocytosis by human neutrophils and monocytes. CHIPS binds directly to the C5aR and formylated peptide receptor (FPR), thereby blocking the C5a- and <i>N</i> -formyl-methionyl-leucyl-phenylalanine (fMLP)-induced calcium responses.	51
SCIN	SCIN inhibits opsonization and phagocytosis by human neutrophils. SCIN binds and stabilizes human C3 convertases, preventing further C3b deposition and activation of the human complement cascade. SCIN also inhibits C5a-induced neutrophil responses.	48
FLIPr	FLIPr is a chemotaxis inhibitor. FLIPr impairs the leukocyte response to FPRL1 agonists by binding directly to host FPRL1.	75
HlgB	HlgB is a bicomponent pore-forming toxin; HlgAB targets chemokine receptors CXCR1, CXCR2, and CCR2, while HlgB targets the complement receptors C5aR and C5L2.	76
LukD/E	LukD/E is a bicomponent pore-forming leukotoxin. LukD/E is less leukotoxic for humans than for rabbits.	77
LukS	LukS induces membrane pore formation in neutrophils, monocytes, and macrophages. The S subunit LukS engages complement receptors C5aR and C5aR2.	78–80
SSL1	SSL1 is a potent human MMP inhibitor.	81
SSL3	SSL3 inhibits TLR2 activation on neutrophils and monocytes. SSL3 prevents TLR2 activation by blocking its ligand binding or engages with the TLR2-lipopeptide complex to prevent further the TLR2 activation signaling cascade.	82, 83
SSL5	SSL5 is a potent human MMP inhibitor.	81
SSL9	Dendritic cells loaded with SSL9 could stimulate a dose-dependent T cell recall response.	84
SEA	SEA is a potent inducer of tolerosome production by enterocytes via unspecific stimulation (superantigen) of intraepithelial lymphocytes (IELs). Tolerosomes seem to be able to manipulate intestinal dendritic cells in response to interpreted orally administered peptides as tolerogens.	85

^aC5aR, C5a receptor; MMP, matrix metalloproteinase; TLR2, Toll-like receptor 2.

icantly higher than that present in the 1st-hour supernatants ($P \leq 0.01$) (Fig. 1F). CHIPS was also observed during the 4th hour of biofilm formation of the M82, RWW50, and Newman strains (Fig. 1C, E, and F), whereas FLIPr was detected only in the supernatants of strain Newman (Fig. 1F). The immune modulators HlgB, Luke, LukD, LukS, SSL1, SSL3, SSL5, SSL9, and SEA were not produced by any of the biofilms of the tested strains under the conditions studied (data not shown). M116 was not positive for the CHIPS gene, and M82 was not positive for the FLIPr gene; therefore, these strains can be seen as negative controls for CHIPS and FLIPr, respectively, along with strain RN6390 (see Table S1 in the supplemental material).

Detection of immune modulator production of *S. aureus* biofilms by mass spectrometry. To confirm the production of immune modulators during the early stages of biofilm formation, we performed mass spectrometry on supernatants representing the 4th hour of biofilm formation of the Newman and RWW50 strains. We could identify the immune modulators CHIPS, SCIN, and FLIPr in these samples (Tables 2 and S2), supporting the results of the competitive Luminex assay (Fig. 1B to F). Other proteins were also identified in the supernatant from the early biofilms, including thermonuclease (Nuc), immunoglobulin-binding protein Sbi, and protein A (Table S2).

Transcription of *scn* and *chp* promoters during biofilm formation. We studied the transcription of both *scn* and *chp* during biofilm formation with the GFP promoter fusion technology. We took earlier described *scn* and *chp* promoter-GFP fusion constructs cloned in strain RN6390 and performed biofilm assays (48). Transcription of the *scn* and *chp* promoters was already detectable after 3 h of biofilm formation, and both were continuously expressed during the entire assay (Fig. 2A). Transcription of the *scn* promoter was significantly higher than that of the *chp* promoter throughout the incubation time ($P < 0.0001$) (Fig. 2).

SCIN inhibits biofilm-induced C5a release. Considering the significant expression of SCIN and, to a lesser extent, that of CHIPS, already during the early stages of biofilm formation, we also studied whether these immune modulators can protect young biofilms from the host innate immune responses. To achieve this aim, we added recombinant SCIN (rSCIN) or recombinant CHIPS (rCHIPS) protein during the 4th hour of biofilm formation to the SCIN- and CHIPS-negative strain RN6390 and detected the generation of human complement component 3a (C3a) and complement 5a (C5a) in the presence of human serum. C5a release in the biofilms spiked with rSCIN was

FIG 1 Biofilm formation and production of the immune modulators SCIN, CHIPS, and FLIPr during the 1st, 2nd, 4th, and 24th hours of biofilm formation of *S. aureus* RN6390, M82 (ST20), M116 (ST239), RWW50 (USA300), and Newman. (A) Biofilm formation of various *S. aureus* strains after 1, 2, 4, and 24 h of incubation in IMDM at 37°C. (B to F) The competitive Luminex assay indirectly monitored the presence of the immune modulators SCIN, CHIP, and FLIPr by determining the percentage of uncaptured IgG specific for these immune modulators in the supernatants of the 2nd, 4th, and 24th hours during biofilm formation of the *S. aureus* RN6390 (B), M82 (C), M116 (D), RWW50 (E), and Newman (F) strains. The *S. aureus* RN6390 strain was used as a negative control for SCIN and CHIPS. The more immune modulator that was present in the supernatant, the lower the amount of signal that was observed, as the presence of uncaptured specific IgG is inversely related to the presence of the immune modulators in the supernatants. Dashed horizontal lines indicate the average MFI for the HPS control. Error bars represent the mean with the SEM ($n = 3$). Statistically significant differences are indicated by asterisks (**, $P \leq 0.01$; ***, $P \leq 0.001$).

significantly inhibited in comparison with that in the biofilms without rSCIN ($P = 0.024$) (Fig. 3A). Addition of rSCIN did not reduce the biofilm-induced release of C3a (data not shown). As expected, the addition of rCHIP had no effect on the biofilm-induced release of C5a (Fig. 3A). Similar experiments were performed for clinical strain M116, an *scn*-negative variant of which was available. In line with the previous observations, more biofilm-induced C5a was generated in sera by the *scn*-negative variant of M116 than by the wild type (Fig. 3B). However, C5a generation was reduced when the supernatant of the wild-type isolate was added to the biofilm of *scn*-negative M116 (Fig. 3B), and a significant reduction of this biofilm-induced C5a was observed with the addition of rSCIN ($P = 0.019$) (Fig. 3B).

DISCUSSION

Using a competitive Luminex and mass spectrometry, we found that during early biofilm formation in the mammalian cell culture medium IMDM, laboratory and clinical strains of *S. aureus* produced immune modulators, including SCIN and, to a lesser extent, CHIPS and FLIPr. Most of the initially selected immune modulators (based on previous studies showing a relation with *S. aureus* colonization *in vivo* [45]) were not produced during the early stages of biofilm formation. This may have been due to the selected culture conditions (medium, surface materials, and timing), which are different from those used in the *in vivo* situation studied previously (45, 47). Nevertheless, our

TABLE 2 Mass spectrometry results for immune modulators produced during the 4th hour of biofilm formation

Strain	Protein	Protein accession no.	Protein identification probability (%) ^a			No. of unique peptides detected ^a			Peptide sequence(s) ^a			Sequence coverage (%) ^a		
			I	II	III	I	II	III	I	II	III	I	II	III
Newman	SCIN	A6QIG6	99.00	99.70		1	1		IYNEIDEALK	IYNEIDEALK		8.62	8.62	
		FLIPr	37.50			1			IKDDGPR			5.26		
	CHIPS		99.50			1			AETVNDLYLVK			9.02		
		A6QG57	100	48.10	100	4	1	5	SSYVINGPGK	MVILTENK	SSYVINGPGK	22.10	5.37	28.20
		A6QIG7	100			3			MVILTENK		MVILTENK	16.80		28.20
RWV50 (USA300)	SCIN	Q2FFF8	99.50	99.60	99.10	1	1	1	IYNEIDEALK	IYNEIDEALK	IYNEIDEALK	8.62	8.62	8.62
	FLIPr	A6QG57	99.50	99.60		1	1		AETVNDLYLVK	AETVNDLYLVK		9.02	9.02	
	CHIPS		100		19.00	3		1	MVILTENK		NSAQFEK	16.80		4.70
		Q2FFF7							NSAQFEK			16.80		
									SSYVINGPGK			16.80		

^aFrom three separate experiments (experiments I to III).

observations confirm the earlier proposed production of immune modulators during the early stages of biofilm formation (11). Additionally, both SCIN transcription and CHIPS transcription were observed during this growth phase using the GFP technology. These findings indicate that SCIN and CHIPS are not exclusively expressed during the exponential growth phase of planktonic bacteria (48) but are also expressed during the early stages of biofilm formation. A study by Zapotoczna et al. (49) described that the 2-component system SaeRS is required for biofilm formation in mammalian cell culture medium, in contrast to classical growth media, such as Trypticase soy broth. Furthermore, SaeRS is known to be involved in the regulation of *S. aureus* immune modulators, such as CHIPS and SCIN (48, 50). SCIN is present in 90% of all clinical *S. aureus* strains (44) and inhibits C3b deposition and thereby C3b-mediated phagocytosis (44). CHIPS is present in 60% of all clinical *S. aureus* strains (51) and inhibits chemotaxis of neutrophils by binding to the C5a receptor and the formylated peptide receptor on neutrophils (48). The growth of bacteria in mammalian cell culture medium is a strategy applied by several groups, considering that *S. aureus* behaves differently in prokaryotic or classical growth medium than in eukaryotic cell culture media (8, 24–29). We hypothesized that mammalian cell culture medium better mimics the situation that bacteria encounter *in vivo* than classic growth medium. Therefore, we focused on the kinetics of immune modulator production and expression during early biofilm formation in a mammalian cell culture medium. The use of mammalian cell culture media, for example, IMDM, has an additional advantage in that it can support both *S. aureus* and human cells during cocubation experiments (52), thus allowing the interactions of biofilms and human cells to be studied under *ex vivo* conditions.

Although we are not the first to find the production of immune modulators by biofilms, little is known about the consequence of their presence in biofilms. SCIN is present in almost all clinical *S. aureus* strains and is a potent C3 convertase inhibitor, which can reduce the complement-mediated phagocytosis of planktonic cells (44, 51). A previous study on planktonic *S. aureus* (53) found that SCIN stabilizes C3 convertase molecules by dimerization of two convertases. These dimers prevent not only opsonophagocytosis (54) but also the formation of C5 convertases. Under normal conditions, the generation of C5 convertase facilitates C5a release. As the C5a convertase is an effective chemoattractant for phagocytes, including neutrophils, toward the site of infection (54, 55), SCIN has a major impact on the ability of the innate immune system to clean up bacteria. In this study, we found that recombinant SCIN could inhibit biofilm-induced C5a release, but we were unable to demonstrate that SCIN reduces C3a release. It seems that in our biofilm setting, SCIN was unable to completely block all three complement activation pathways, resulting in major C3a release. In a previous study by Rooijackers et al. (44), addition of 0.3 $\mu\text{g/ml}$ of SCIN almost completely

FIG 2 Kinetics of *chp* and *scn* transcription of *S. aureus* during the 4th and 5th hours of biofilm formation. (A) The levels of transcription of the *chp* and *scn* promoters during the 4th and 5th hours are presented, along with the GFP signal of strain RN6390 with a constitutive phage repressor promoter-GFP construct (PrP-GFP), which indirectly reflects bacterial growth. Data are presented as the mean with the SEM ($n = 3$). (B to D) The promoter activities of the PrP-GFP control (B), the *chp*-GFP construct (C), and the *scn*-GFP construct (D) after 4 h of biofilm formation, determined by fluorescence microscopy, are visualized, and the figures present representative results of three separate experiments.

blocked the alternative pathway, but concentrations up to 10 μ g/ml were needed to halve the amount of complement activation via the classic and lectin pathways. We did see blocking of biofilm-induced C5a after addition of SCIN, indicating that SCIN indeed facilitated the dimerization of C3 convertases in the biofilm (53) at a certain level. Furthermore, because spontaneous activation of complement can easily occur under *in vitro* conditions (56, 57) and C3 activation products have half-lives of a few hours (58), it is hard to specifically pinpoint when C3 is activated. On the other hand, the released C5 activation products can more accurately be determined, as their half-lives are much shorter, i.e., a few minutes (59). Therefore, our data indicate that C5a is a better marker for *de novo* biofilm-induced complement activation, allowing study of the impact of different (bacterial) components on this part of the human innate immune system.

During the early stages of biofilm formation, *S. aureus* is able to manipulate the immune system, which potentially facilitates the survival of the biofilm-forming bacteria, leading to the establishment of a biofilm. We anticipate that besides CHIPS, SCIN, and FLIPr, other immune modulators, such as extracellular fibrinogen binding protein (Efb) (60) and surface protein serine-aspartate repeat protein E (SdrE) (61), might be involved in immune avoidance during biofilm formation as well. However, under the conditions under which we performed the experiments and with the strains studied, we

FIG 3 Inhibition of biofilm-induced C5a release by SCIN. Human serum was added to 3-hour-old biofilms of the SCIN-negative strain RN6390 (A) and clinical isolate M116 (WT) and its *scn*-negative variant (Δ scn) (B) in the presence or absence of rSCIN (A, B), rCHIPS (A), or the supernatant (sup) of the wild-type M116 strain (B). After 30 min of incubation at 37°C, the released C5a was measured using an ELISA. Data from three separate experiments are presented as the mean value and SEM. Statistically significant differences are indicated by asterisks (*, $P \leq 0.05$; ***, $P \leq 0.001$).

could not confirm this. Nevertheless, our findings form a stepping stone toward elucidating the role of immune modulators in the establishment of biofilms *in vivo*. The setup used in this study not only provides a rapid screening method to identify factors produced during the early stages of biofilm formation but also allows us to determine the roles of these factors in the establishment of a biofilm, thereby presenting opportunities to develop preventive strategies. Biofilms of *S. aureus* are an important clinical problem for several reasons (62), and helping the immune system to neutralize biofilm-forming bacteria will be of enormous benefit to human health care.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The clinical and laboratory *S. aureus* isolates used in this study are listed in Table 3. *S. aureus* confirmation was performed by Slidex Staph Plus (bioMérieux, Zaltbommel, The Netherlands) agglutination assays. All wild-type *S. aureus* strains were grown on Trypticase soy agar (TSA) with 5% sheep blood (Becton Dickinson, Breda, The Netherlands) overnight at 37°C. The *scn*-negative variant of M116 was acquired by culturing the wild-type strain on TSA several times overnight at 37°C until beta-hemolysis-producing colonies appeared. This is indicative of the loss of *scn*, which is carried by beta-hemolysin (*hly*)-converting bacteriophages (β C- ϕ S) (63). The absence of *scn* was confirmed by PCR (see Table S1 in the supplemental material). The GFP construct-containing strains were plated on TSA supplemented with 10 μ g/ml chloramphenicol (Oxoid Limited, Basingstoke, UK).

Immune modulator gene determination. All strains were tested for the presence of the immune modulator genes *scn*, *chp*, *flr*, *hlgB*, *lukE-lukD*, *lukS*, *ssl1*, *ssl3*, *ssl5*, *ssl9*, and *sea* (Table S2) according to a previously described PCR protocol (64).

Biofilm mass assessment. Biofilm formation was assessed *in vitro* using a dynamic, microtiter plate biofilm formation assay as previously described (37, 65–67) with slight modification. *S. aureus* bacteria cultured overnight on TSA were suspended in 4 ml of 0.9% NaCl to reach an optical density at 660 nm (OD₆₆₀) of 2.0 $\pm$ 0.2. One microliter of this suspension was added to 199 μ l Iscove's modified Dulbecco's medium (IMDM; Gibco, Bleiswijk, The Netherlands), an iron-free growth culture medium, in separate wells of a sterile flat-bottom, 96-well polystyrene tissue culture plate (CellStar; Greiner Bio-One, Alphen aan de Rijn, The Netherlands) (67). The plates were incubated for either 1, 2, 4, or 24 h under 150-rpm orbital shaking at 37°C. At these time points, the biofilms formed were washed once with 200 μ l sterile water, air dried for 30 min at room temperature, and then stained for 2 min with 50 μ l of 1% crystal violet (Sigma-Aldrich, Zwijndrecht, The Netherlands). Excess crystal violet was removed by washing the plates

TABLE 3 *S. aureus* strains used in this study

Strain	Genetic background	Description	Source or reference
M82	ST20	Isolated from a patient with osteomyelitis in Indonesia	This study
M116	ST239	Isolated from a patient with osteomyelitis in Indonesia	This study
M116 β	ST239	Δ scn, SCIN (scn)-negative variant of M116	This study
RWW50	ST8, USA300	Clinical isolate from the collection of MMIZ, Erasmus MC, Rotterdam, The Netherlands	W. van Leeuwen
Newman	ST8	Wild type	86
RN6390	ST8	Wild type, GFP-negative control	87
RN6390 PrP-GFP	ST8	GFP-positive control with a Page repressor promoter cloned into the EcoRI-XbaI site of pACL1484	71
RN6390 scn-GFP	ST8	scn promoter cloned into the EcoRI-XbaI site of pACL1484	48
RN6390 chp-GFP	ST8	chp promoter cloned into the EcoRI-XbaI site of pACL1484	48

five times with 200 μ l of sterile water. The biofilms were dissolved in 200 μ l extraction solution consisting of 50% distilled H₂O, 40% ethanol (Sigma-Aldrich), and 10% acetic acid (Sigma-Aldrich). Lastly, to determine the biofilm mass, the absorbance of crystal violet was measured at OD₄₉₀ in a microplate reader (Epoch 2 microplate reader; BioTek Instruments, Inc., Winooski, VT, USA).

Collecting biofilm supernatants. To determine the production kinetics of the selected immune modulators during biofilm formation, we cultured *S. aureus* strains RN6390 and Newman and clinical strains M82 (ST20), M116 (ST239), and RWW50 (USA300) as described above. We cultured the biofilm for either 1, 3, or 23 h. At these time points we discarded the supernatant to remove all nonattached or planktonic bacteria, washed the biofilm with 200 μ l IMDM once, and added 200 μ l of fresh IMDM. Incubation was then continued under the same conditions for 1 h, after which the supernatants were collected and labeled as either the 2nd-, 4th-, or 24th-hour biofilm supernatants. All individual supernatants from all time points and all different strains were filtered through a 0.2- μ m-pore-size sterile filter (Whatman GE Healthcare Life Sciences, Little Chalfont, UK) and stored at -20°C until further usage. After 1 h of biofilm formation, supernatant was collected from all strains and used as a reference control.

Semiquantification of immune modulators in biofilm supernatants by competitive Luminex assay. Thirty microliters of each biofilm supernatant collected as described above was diluted in 30 μ l of phosphate-buffered saline (PBS)-BN buffer (PBS containing 1% bovine serum albumin [Roche, Mannheim, Germany] and 0.05% sodium azide [Sigma] [pH 7.4]). Then, the competitive assay was performed as described previously (37, 46). With the competitive Luminex assay, we could assign semiquantitatively the amount of antigens present by determining the ratio of the amount of antibodies bound to the antigens to the amount of unbound antibodies after preexposing serum antibodies to a liquid containing an unknown amount of free antigens. Briefly, 30 μ l of the diluted biofilm-derived supernatants was incubated separately with 30 μ l of 1/200-diluted human pooled serum (HPS) (37, 46). The mixture was incubated at 22°C in a U-shaped 96-well microplate (Greiner Bio-One) placed within a ThermoStat plus block (Eppendorf, Nijmegen, the Netherlands) for 35 min with continuous shaking at 800 rpm. Next, 50 μ l of this mixture was directly used for the simultaneous semiquantification of the noncaptured IgGs specific for *S. aureus* biofilm-derived immune modulators using Luminex bead-based flow cytometry (Luminex Corporation, Austin, TX, USA). The antigens used in this assay were CHIPS, SCIN, FLIPr, HlgB, LukD, LukE, LukS, SSL1, SSL3, SSL5, SSL9, and SEA (45). All antigens were coupled to MagPlex beads (Luminex Corporation) according to the manufacturer's protocol. The antigen concentration used for coupling was 2 μ g per antigen per 10^6 MagPlex beads, as optimized by Hansenová Maňásková et al. (68). The beads were diluted to a final concentration of 1,500 beads/ μ l. Negative-control beads (beads to which no antigen was added during coupling) were included in all assays. The Luminex assay was performed as described previously (69) with one modification; that is, we measured 50 beads per region (68). All samples were analyzed on a Luminex BioPlex 200 system using Bio-Plex Manager software, version 6.1 (Bio-Rad Laboratories, Inc., USA).

All data are based on three separate experiments. The median fluorescence intensity (MFI) values from these triplicate experiments were averaged if the coefficient of variation was less than 25%, and the standard error of the mean (SEM) was calculated. For each sample and each individual antigen, we calculated the proportion (in percent) of uncaptured IgG specific for the individual immune modulators using the formula $Y = (A/B) \times 100$, in which A is the unbound IgG specific for that specific immune modulator present in the HPS-biofilm supernatant mix, and B is the level of immune modulator-specific IgG present in untreated HPS. Y can be considered a semiquantitative measurement of the antigen-specific antibody absorption from HPS by the biofilm supernatant, thus indirectly reflecting the immune modulators present in the biofilm supernatant (47). Statistically significant differences between the time points were calculated by comparing the proportions for the 2nd-, 4th-, and 24th-hour time points and using the proportion for the 1st hour as the reference control.

Identification of the immune modulators by mass spectrometry. One hundred microliters of the supernatant derived from the 4th hour of biofilm formation of the RWW50 (USA300) and Newman strains was precipitated at 1:10 (vol/vol) with ice-cold acetone before further sample preparation. Samples were centrifuged for 10 min at $18,407 \times g$ at 4°C , and the pellets were subsequently washed twice with 50 μ l of cold acetone. Then, the pellets were air dried for 30 min and dissolved in 10 μ l of 50 mM NH₄HCO₃. Solution digestion was performed according to an adjusted protocol described by Dekker et al. (70). Ten

microliters of 0.2% RapiGest surfactant (Waters Corporation, Milford, MA, USA), which was previously diluted in 50 mM NH_4HCO_3 , was added into 10 μl sample. This mixture was then reduced with 5 mM dithiothreitol (DTT; Thermo Fisher Scientific, Waltham, MA, USA) at 60°C and 450 rpm for 30 min. After this mixture was cooled to room temperature, it was alkylated in the dark with 15 mM iodoacetamide (Thermo Fisher Scientific) at ambient temperature for 30 min and digested overnight with 1:100 (wt/wt) trypsin (Promega Corporation, Fitchburg, WI, USA) at 37°C and 450 rpm. Five percent trifluoroacetic acid was added to obtain a final concentration of 0.5% trifluoroacetic acid ($\text{pH} < 2$). After 45 min of incubation at 37°C and 450 rpm, the samples were centrifuged at $13,000 \times g$ for 10 min, and the resulting supernatant was used for liquid chromatography-mass spectrometry. Mass spectrometry and data analyses were performed as described elsewhere (70). A search against the uniprot_sprot_v151112 database was performed (selected for *Bacteria*, 332,280 entries).

Measurement of CHIPS and SCIN immune modulator transcription. To show the transcription kinetics of the immune modulators CHIPS and SCIN during biofilm formation, we selected all GFP-containing *S. aureus* strains (Table 3) and generated *in vitro* biofilms by the method described above. After 3 h of incubation at 37°C, the growth medium was replaced with 200 μl of fresh IMDM. The biofilms were then incubated in a FLUOstar Optima microplate fluorescence reader (BMG Lab Technologies, Chicago, IL, USA) at 37°C with periodic rotational shaking at 150 rpm. The accumulation of fluorescence, as a measurement for gene transcription, was determined (excitation, 485 nm; emission, 520 nm; gain setting, 1,738) automatically every 5 min during the 4th and 5th hours of biofilm formation. The MFI of the *chp* promoter-GFP (CHIPS) and *scn* promoter-GFP (SCIN) were compared to the MFI of the GFP-positive control (strain RN6390 carrying a strong constitutive phage repressor promoter-GFP fusion construct [Table 3] as, previously described by Rooijackers et al. [71]). For visualization of the fluorescence emission, 4-h-old biofilms were mounted onto an Olympus IX51 fluorescence microscope (Olympus Nederland B.V., Zoeterwoude, The Netherlands) with a $\times 40$ magnification. Before imaging, planktonic cells were removed by refreshing the medium.

Measurement of complement activation. One of the critical steps of complement activation is the formation of human complement 3 (C3) convertases, which subsequently cleave more C3 to C3a and C3b (44, 72, 73). C3b, along with other components, forms C5 convertase. This C5 convertase is responsible for the formation of C5a (chemoattractant) and C5b from C5 (74), which, among others, is a crucial point in one of the tasks of the complement activation cascade for fighting microorganisms (44, 72, 73). Therefore, to study the ability of SCIN to modulate complement activation during the early stages of biofilm formation, 3-h-old biofilms of SCIN- and CHIPS-negative strain RN6390 (Table S1) were washed, refreshed with 100 μl IMDM, and incubated for 30 min at 37°C. One hundred microliters of diluted human serum in IMDM (1:5,000 for the C3a assay and 1:100 or 1:200 for the C5a assay) was added to these biofilms in the presence or absence of 3 $\mu\text{g}/\text{ml}$ either recombinant SCIN (rSCIN) or recombinant CHIPS (rCHIPS). After a 30-min incubation period at 37°C, the supernatants were harvested, filtered through a 0.2- μm -pore-size sterile filter (Whatman GE Healthcare Life Sciences), and then processed according to the protocol of the human C3a and C5a enzyme-linked immunosorbent assay (ELISA) kit II (OptEIA; BD, San Jose, CA, USA). The C3a and C5a concentrations were measured at OD_{450} (λ correction at OD_{570}) with a microplate reader (Epoch 2 microplate reader; BioTek). To study the role of SCIN in C5a activation of a clinical strain, similar kinds of studies were performed using wild-type strain M116 and the *scn*-negative variant of strain M116 (Table 3). For complementation, rSCIN or the 4th-hour biofilm supernatants of the wild type were added to the biofilm of the *scn*-negative variant.

Data analysis. Statistical analysis was performed with the Prism (version 5.0) software package (GraphPad Software, San Diego, CA, USA) and Microsoft Excel 2010 software. Data were analyzed with an unpaired *t* test or one-way analysis of variance; a *P* value of ≤ 0.05 was considered statistically significant. All experiments were independently repeated three times, and data are presented as the mean (SEM) or as the median (range).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/IAI.00352-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.1 MB.

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