Staphylococcus aureus Proteases Degrade Lung Surfactant Protein A Potentially Impairing Innate Immunity of the Lung

Tomasz Kantyka^a Krzysztof Pyrc^{a,c} Milosz Gruca^a Jan Smagur^a Karolina Plaza^a
Krzysztof Guzik^b Sławomir Zeglen^d Marek Ochman^d Jan Potempa^{a,e}

Departments of ^aMicrobiology and ^bImmunology, Faculty of Biochemistry, Biophysics and Biotechnology, and ^cMalopolska Centre of Biotechnology, Jagiellonian University, Krakow, and ^dDepartment of Cardiac Surgery and Transplantology, Silesian Center for Heart Diseases, Zabrze, Poland; ^eCenter for Oral Health and Systemic Diseases, School of Dentistry, University of Louisville, Louisville, Ky., USA

Key Words

Bacteriology · Host defense · Proteinases

Abstract

The pulmonary surfactant is a complex mixture of lipids and proteins that is important for respiratory lung functions, which also provides the first line of innate immune defense. Pulmonary surfactant protein-A (SP-A) is a major surfactant component with immune functions with importance during *Staphylococcus aureus* infections that has been demonstrated in numerous studies. The current study showed that *S. aureus* can efficiently cleave the SP-A protein using its arsenal of proteolytic enzymes. This degradation appears to be mediated by cysteine proteases, in particular staphopain A (ScpA). The staphopain-mediated proteolysis of SP-A resulted in a decrease or complete abolishment of SP-A biological activity, including the promotion of *S. aureus* phagocytosis by neutrophils, aggregation of Gram-negative bacteria and bacterial cell adherence to epithelium. Significantly, ScpA has also efficiently degraded SP-A in complete bronchi-alveolar lavage fluid from human lungs. This indicates that staphopain activity in the lungs is resistant to protease inhibitors, thus suggesting that SP-A can be cleaved in vivo.

Collectively, this study showed that the *S. aureus* protease ScpA is an important virulence factor that may impair innate immunity of the lungs.

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Introduction

The pulmonary surfactant is a complex mixture of lipids and proteins that forms a thin film at the air-liquid interface of the lung alveoli, which is essential for respiratory functions and gas exchange [1]. Apart from its function in the gas exchange process, the surfactant also provides the first line of innate immune defense in the lungs. Its mechanism of action relies on the enhancement of microbial neutralization and the clearance of microorganisms, as well as attenuation of potentially harmful inflammatory responses [2–9]. The major components responsible for this immune defense function are the pulmonary surfactant proteins, i.e. SP-A, SP-B, SP-C and SP-D. SP-A and SP-D, which are large proteins structurally

Tomasz Kantyka and Krzysztof Pyrc contributed equally to this work.

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Fax +41 61 306 12 34
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Dr. Krzysztof Pyrc
Department of Microbiology, Faculty of Biochemistry
Biophysics and Biotechnology, Jagiellonian University
Gronostajowa 7, PL–30–387 Krakow (Poland)
E-Mail k.a.pyrc@uj.edu.pl

and functionally related to hydrophilic proteins that belong to a subgroup of mammalian lectins known as 'collectins' (together with mannose-binding lectin), whereas SP-B and SP-C are small hydrophobic proteins [10].

SP-A is the major constituent of the pulmonary surfactant where it is involved in the organization of large surfactant phospholipid aggregates that line the alveolar surface and acts as an opsonin for colonizing bacteria [11–13]. SP-A is incorporated in the tubular myelin fraction of the pulmonary surfactant that covers the alveolar lining fluid of the distal airway epithelium, which can reorganize and expose SP-A, allowing it to bind to pathogens [14, 15]. As a consequence, immune cells present in the alveolus of the lung, such as macrophages, neutrophils and monocytes, can easily recognize pathogens via binding to SP-A-opsonized bacteria. SP-A binds pathogens by a C-terminal lectin-like domain and its collagen-like N-terminal domain is involved in the oligomerization of SP-A molecules [16]. SP-A aggregates in the presence of Ca^{2+} ions, which may facilitate its binding to multiple epitopes exposed on bacterial surfaces [17]. The importance of SP-A in innate defense mechanisms has been demonstrated in vitro and in vivo. SP-A-deficient mice are highly susceptible to viral and bacterial infections caused by *Staphylococcus aureus*, group B streptococcus, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Pneumocystis jiroveci* and the respiratory syncytial virus [2, 18–23].

S. aureus is a Gram-positive bacterium that persistently colonizes ~20% of the human population [24]. This high prevalence is reflected in the role of *S. aureus* as an important pathogen responsible for various illnesses in humans and animals worldwide, including respiratory tract, musculoskeletal, bloodstream, skin and soft tissue infections [25–27]. Extracellular proteases produced by *S. aureus* appear to be essential for successful infections. The most abundant secreted enzymes are staphopains A (ScpA) and B (cysteine proteases), V8 protease (serine protease) and aureolysin (metalloprotease) which activate themselves in a proteolytic cascade [28]. These proteases interact with the host tissue components in vitro to modulate host defense mechanisms [29, 30] and likely increase bacterial survival [31–38].

Previous reports demonstrate that the SP-A protein is also important for *S. aureus* clearance. Specifically, SP-A binds the extracellular adhesion protein on the staphylococcal surface and links the complex to SP-A receptor 210 (SP-R210) and scavenger class A receptors (SR-A) on macrophages. In this way SP-A facilitates *S. aureus* clearance from the infected lungs by phagocytosis [23].

The current study investigated the effect of *S. aureus* extracellular proteases, including ScpA, staphopain B, V8 protease and aureolysin, on the integrity and function of SP-A. We found that among tested staphylococcal proteases ScpA most efficiently degraded SP-A and abolished the antibacterial activity of this surfactant protein.

Materials and Methods

Cells and Bacteria

Polymorphonuclear cells (neutrophils) were isolated from erythrocyte-rich blood by sedimentation in 1% polyvinyl alcohol solution (Merck) for 20 min at room temperature. Neutrophils were collected from the upper layer and contaminating erythrocytes were lysed by a 20-second incubation in demineralized water. Pappenheim staining indicated that isolated cells were at least 90% homogenous. Neutrophils were used immediately after harvesting. Human lung carcinoma A549 cells (ATTC CCL-185) were cultured in D-MEM media (PAA) supplemented with 10% of FBS and non-essential amino acids (PAA).

Well-characterized *S. aureus* strains: Newman (human isolate; kindly provided by T.J. Foster), LAC (Los Angeles County; community-associated, methicillin-resistant strain) and 8325-4 (laboratory strain; high expression of proteolytic enzymes), a kind gift from Lindsey Shaw, were cultured in tryptic soy broth (TSB) liquid medium (Sigma-Aldrich). *Salmonella* spp. (kindly granted by Dr. Kinga Wójcik) was cultured in Luria-broth liquid media (Sigma-Aldrich). In all cases, the bacteria concentration was assessed by optical density measurements in liquid media (OD_{600} or OD_{660}) or by scoring the number of colonies on solid media (colony-forming unit number; cfu).

Isolation of *S. aureus* Extracellular Proteases

Both staphopains and V8 protease were purified from the *S. aureus* BC10 V8 strain, as described previously [39]. Staphopain B and V8 protease were purified on a Q Sepharose FF (GE Healthcare) column at pH 5.5. Fractions containing proteolytic activity were further purified on a Phenyl Sepharose High Performance column (GE Healthcare). ScpA was purified using CM Sepharose FF (Amersham-Pharmacia) chromatography followed by high-resolution ion exchange chromatography on a MonoS HR 5/5 column (GE Healthcare).

Aureolysin was purified on a DEAE-Sepharose column (GE Healthcare). Resulting fractions exhibiting EDTA-sensitive proteolytic activity were pooled and dialyzed against 50 mM Tris-HCl pH 7.6. All proteases were aliquoted and stored at -20°C .

Enzymatic activity of ScpA, staphopain B in purified samples was determined by titration using azocol substrate with macroglobulin and staphostatin B, respectively. Activities of aureolysin and V8 protease were assumed to be 100%.

Proteolytic Processing of Surfactant Protein A

Cysteine proteases (ScpA and staphopain B) were activated by incubation in buffer containing 2 mM DTT, 50 mM Tris-HCl, pH 7.6 and 5 mM EDTA. SP-A was incubated in the presence of bacterial proteases at 37°C for 90 min. Furthermore, samples containing SP-A were mixed with SDS-PAGE sample buffer, separated on

polyacrylamide gel and electrotransferred onto a nitrocellulose membrane for Western blot analysis with IgM polyclonal mouse antibodies against SP-A proteins (Millipore MAB3270) and rabbit α -mouse HRP-conjugated antibody (BD Pharmingen), as primary and secondary antibodies, respectively. A signal was developed using the ECL kit (Thermo Scientific).

To determine whether the degradation of SP-A by ScpA is time dependent, the protein was incubated in presence of 50 nM ScpA for 180 min at 37°C, and after 12-, 30-, 45-, 60-, 90-, 120- and 180-min samples were withdrawn into the SDS-PAGE sample buffer and immediately boiled to stop the reaction. Analysis with Western blot was carried out as described above.

Degradation of SP-A in Bronchoalveolar Lavage in Presence of S. aureus Proteases

S. aureus (strains LAC, Newman and 8325-4) were cultured overnight in liquid media, supplemented with casein (5%) and β -glycerophosphate (5 g·l⁻¹). Bacteria were pelleted (4,000 g, 20 min) and supernatants were tested for their enzymatic activity.

Human bronchoalveolar lavage fluid (BALF) was obtained from human lungs rejected from the lung transplant program of the Silesian Center for Heart Diseases. Briefly, conductive airways were perfused with buffered solution (5 mM Tris, pH 7.4, 150 mM NaCl). Samples were cleared by centrifugation, aliquoted and frozen at -80°C. In order to analyze the degradation of SP-A in BALF, 35 μ l of BALF was mixed with 15 μ l of bacterial culture supernatants and incubated for 6 h at 37°C. As a control, 35 μ l of BALF was incubated with 15 μ l of media. The reaction was stopped by addition of 15 μ l of SDS-PAGE sample buffer (4% SDS, 60% glycerol, 0.3 M Tris, 0.01% Coomassie Brilliant Blue G-250, 50 mM DTT) and by boiling (5 min, 99°C). Proteins were separated by SDS-PAGE electrophoresis in the Schagger-von Jagow system. Subsequently, samples were transferred onto the PVDF P⁵⁰ membrane (Millipore) for Western blot analysis of the SP-A protein. The membrane was blocked in 3% skimmed milk (Fluka) at 4°C overnight and a signal was developed as described above.

Aggregation Assay

Salmonella spp. was cultured overnight in Luria broth media. Bacteria were pelleted by centrifugation (4,000 g, 20 min) and washed three times with sterile 1× PBS. A bacteria cell pellet was re-suspended in sterile 1× PBS to reach final OD₆₆₀ of 1. SP-A protein was added to the suspension to reach the final concentration of 7.5 μ g·ml⁻¹. The suspension was incubated at 37°C and the bacterial aggregation was assessed by means of sedimentation rate, employing the optical density measurements (λ = 660 nm) every 15 min for 1 h.

Adhesion Assay

A549 cells were seeded on 24-well plates 48 h prior to the experiment in order to reach full confluency. On the day of the experiment cells were washed three times with sterile 1× PBS and cooled down on ice. The Newman *S. aureus* strain was cultured in TSB (Sigma-Aldrich) overnight. The bacteria were washed three times with 1× PBS and re-suspended in fresh D-MEM media to reach the final concentration of 10⁶ cfu·ml⁻¹. The suspension was further incubated for 30 min at 4°C with SP-A (25 μ g ml⁻¹), but beforehand it was incubated with ScpA (50 nM) or with control buffer.

S. aureus pre-incubated with SP-A protein (treated or untreated with staphopains) or control media was transferred onto A549 cells and incubated for 60 min at 4°C. After that time the bacteria suspension was removed and cells were washed three times with pre-cooled sterile 1× PBS. The cells and bacteria were detached from the plate surface with 0.5 × trypsin-EDTA solution (PAA) at room temperature and serial dilutions were seeded onto tryptic soy agar plates.

Phagocytosis of S. aureus by Human Neutrophils

S. aureus was cultured in TSB media (Sigma-Aldrich) overnight. After that the bacteria were centrifuged and washed three times with 50 ml of 1× PBS. Pelleted bacteria were further re-suspended in 2 ml of 1× PBS and the optical density of bacterial cells was assessed (λ = 600 nm). Cell count (cfu) was estimated based on previously developed standard curves and bacterial concentration was adjusted to 10⁹ cfu·ml⁻¹. To enable analysis of phagocytosis by flow cytometry, bacteria were incubated for 1 h at 37°C in 1× PBS containing 0.1% of fluorescein isothiocyanate (FITC; Sigma-Aldrich). Non-bound FITC was washed out with 1× PBS in order to reduce the background staining. Labeled bacteria were further incubated with the SP-A protein (25 μ g·ml⁻¹) for 45 min at 37°C in 5-ml round-bottom polystyrene tubes (Becton Dickinson). After the incubation, 25 μ l of *S. aureus* (10⁹ cfu·ml⁻¹) or control media was mixed with the 250 μ l of neutrophil suspension (5 × 10⁵ cells·ml⁻¹) and incubated at 37°C for 60 min. Incubation was stopped by addition of 1 ml of ice-cold 1× PBS supplemented with BSA (0.1%). Cells were centrifuged (110 g, 5 min) and washed with 1× PBS to separate unbound bacteria. Eventually, cells were re-suspended in 1× PBS supplemented with BSA (0.1%). To exclude adherent but not internalized *S. aureus* from analysis, trypan blue was used as previously described [5]. FACS analysis was conducted on a FACScan machine (Becton Dickinson).

Statistical Analysis

All experiments were repeated at least three times and results are expressed as mean $\pm$ SD. To determine the significance of obtained results, a comparison between groups was made using Student's t test. p values < 0.05 were considered significant.

Results

Proteolytic Cleavage of SP-A by S. aureus Extracellular Proteases

This study assessed the ability of extracellular proteases of *S. aureus* to degrade SP-A and inactivate antimicrobial activities of this protein. To this end we have first incubated SP-A with various concentrations of *S. aureus* proteases (ScpA, staphopain B, V8 protease and aureolysin), and analyzed the SP-A protein degradation by SDS-PAGE.

The results clearly showed that SP-A was cleaved efficiently only by ScpA. Importantly, the ScpA concentration sufficient for total degradation of the SP-A protein

Fig. 1. Analysis of SP-A protein processing by *S. aureus* enzymes. SDS-PAGE (**a**) and Western blot (**b**) analysis of protease-mediated SP-A degradation (90 min, 37°C). Multiple bands visible on the Western blot and SDS-PAGE result from the fact that the SP-A protein forms multimeric complexes stable enough to survive the denaturing conditions. Discrepancy in intensity of bands between the SDS-PAGE gels and Western blots results from differ-

ences in antibody affinity towards particular multimeric SP-A forms and cleavage products. Names presented on the left side of the figure denote the enzyme used. Under each panel information on presence/absence of SP-A and concentration of the enzyme is given. Additional visible bands represent monomers and multimers of the SP-A protein. The results shown are representative of at least four independent experiments.

Fig. 2. Time-dependent degradation of SP-A protein by *S. aureus* ScpA. SP-A protein was incubated with ScpA (**a**) or with a control buffer (**b**), as described in the Methods section. Information on presence/absence of SP-A and ScpA in each well is denoted beneath the panel. Additionally, visible bands represent monomers and multimers of the SP-A protein. The lane containing SP-A not treated with ScpA is provided in the right panel. The results shown are representative of at least three independent experiments.

was in the nanomolar range <50 nM. Incubation with staphopain B only resulted in partial cleavage of the protein at high enzyme concentrations, thereby reducing its possible *in vivo* function. No cleavage of SP-A was observed during incubation with V8 protease and aureolysin (fig. 1) even at high protease concentrations and with an extended incubation time. The complete degradation of the SP-A protein by ScpA was visible after 45 min of incubation (fig. 2).

The ability of *S. aureus* proteases to specifically degrade SP-A protein was also assessed in an *ex vivo* system to confirm that the observed effect was not a result of structural alteration associated with protein purification procedure, or the storage and reaction conditions. Briefly, freshly collected BALF was incubated with supernatants from overnight cultures of various *S. aureus* strains, including LAC, Newman and 8325-4. Western blot analysis clearly showed that the SP-A found in human BALF was degraded after incubation with *S. aureus* bacterial supernatants (fig. 3).

Proteolytic Cleavage of Surfactant Proteins Decreases SP-A-Mediated Bacterial Aggregation

S. aureus lung infections have frequently been found to be coincident with infections by Gram-negative bacteria [40–44]. This suggests that SP-A degradation by ScpA may affect the clearance of other pathogens during polymicrobial pneumonia. A previously described model, based on *Salmonella* spp., was employed to test whether the ScpA-mediated degradation of SP-A affected this surfactant protein activity toward Gram-negative bacteria.

Fig. 3. Western blot analysis of SP-A degradation in BALF in the presence of *S. aureus* culture supernatants. BALF was incubated with supernatants from cultures of *S. aureus* strains LAC, 8325-4 and Newman as described in the Methods (part A). As controls, bacterial supernatants are also included (part B). The distinct band in the lane corresponding to the *S. aureus* Newman strain represents staphylococcal protein A. The results shown are representative of at least three independent experiments.

Incubation of *Salmonella* spp. in the presence of the SP-A protein resulted in the rapid sedimentation of bacterial aggregates. However, this effect was severely reduced if the SP-A protein was pre-incubated with *S. aureus* ScpA protease (fig. 4). An assay using a specific synthetic cys-

Fig. 4. Decrease in Gram-negative bacteria aggregation following SP-A incubation with ScpA. Human SP-A pre-incubated with ScpA was added to the *Salmonella* spp. culture. Change of absorbance due to the bacterial clumping/sedimentation was monitored. Results are presented as the percent change in the optical density at $\lambda = 660$ nm for the average values from three independent experiments. Buffer denotes the reaction buffer. E64 and SAF (staphostatin A) denote universal inhibitor of papain-like cysteine proteases and the proteinaceous, ScpA-specific inhibitor, respectively.

teine protease inhibitor (E64) and a natural ScpA inhibitor (staphostatin A) was conducted to determine whether the observed effect was the sole result of staphopain-mediated cleavage. The results suggested a direct link between the proteolytic activity of ScpA and the loss of function of the SP-A protein.

Proteolytic Processing of Surfactant Proteins Increases Adherence of S. aureus to Epithelial Cells

S. aureus binds to eukaryotic cells and it is possible that surfactant proteins may interfere with bacterial adherence to the epithelium, hindering their ability to colonize the respiratory tract [45]. This study analyzed the effect of the SP-A protein on the ability of *S. aureus* to adhere to A549 cells (ATCC CCL-185; a human lung carcinoma-derived cell line). SP-A concentrations up to $5 \mu\text{g}\cdot\text{ml}^{-1}$ did not affect the adherence of *S. aureus* to epithelial cells [46]; therefore, in this study we tested SP-A activity at higher concentrations, but still well below the physiological range ($50\text{--}100 \mu\text{g}\cdot\text{ml}^{-1}$) of this protein concentration in the lungs. Already at $25 \mu\text{g}\cdot\text{ml}^{-1}$ SP-A decreased bacterial staphylococci adherence to the A549 cell monolayer by $\sim 40\%$. Pre-incubation of SP-A with ScpA impeded this

process and the number of bacteria attached to eukaryotic cells was restored to $>80\%$ in relation to the positive control (fig. 5). This experiment clearly shows that ScpA may influence not only the direct effect of SP-A on bacteria, but also its interaction with host cells. Considering the importance of such findings, the subsequent analysis was designed to evaluate the ability of ScpA to impair the well-described role of SP-A in innate immunity, specifically pathogen phagocytosis by neutrophils.

Proteolytic Processing of Surfactant Proteins Impairs Bacterial Phagocytosis by Neutrophils

SP-A protein mediates bacterial phagocytosis by neutrophils [5, 23]. Flow cytometry was used to confirm this and it was found that the ratio of neutrophils actively binding and internalizing *S. aureus* bacteria was drastically increased in the presence of SP-A (fig. 6). However, the pre-incubation of SP-A with ScpA resulted in the loss of the ability to enhance phagocytosis (fig. 1), as shown by the change in the ratio between neutrophils which internalized FITC-labeled *S. aureus* and the whole neutrophil population. ScpA totally abrogated the effect of SP-A on bacteria engulfment as revealed by restoration of the

Fig. 5. Increase in *S. aureus* adherence to A549 cells following SP-A incubation with ScpA. *S. aureus* adherence to human epithelial cell line was assessed as described in the Methods section. Bacteria incubated with SP-A, SP-A/ScpA, with reaction buffer without (Buffer_{Ctrl}) and with reducing agents (Buffer_{DTT}) were inoculated onto the cell monolayer. Following the incubation and removal of unattached bacteria, adherent bacteria were scored. Data are presented as percent change in the number of adherent bacteria, as compared to the sample incubated with buffer without the reducing agents (Buffer_{Ctrl}). The results shown are average values from four independent experiments. The statistical significance of differences in *S. aureus* adherence to eukaryotic cells when compared with a control is indicated: ** $p < 0.005$. n.s. = Not significant.

Fig. 6. Decrease in *S. aureus* phagocytosis by neutrophils after SP-A incubation with ScpA. FITC-labeled bacteria pre-incubated with SP-A, SP-A plus ScpA or ScpA alone were incubated with human neutrophils. Subsequently, bacteria that were not bound were removed, fluorescence derived from non-internalized bacteria was quenched using trypan blue and cells were analyzed by flow cytometry. Results shown are average values from three independent experiments and represent percentage of cells that internalized *S. aureus* bacteria. The statistical significance of differences in *S. aureus* adherence to eukaryotic cells when compared with a control is indicated: * $p < 0.05$; ** $p < 0.005$.

neutrophil phagocytosis efficiency to the control level. Clearly, ScpA had no effect on the basal level of *S. aureus* of clearance by neutrophil. This observation indicates that staphylococcal protease targeted exclusively phagocytosis dependent on the SP-A-opsonizing activity.

Discussion

Three major catalytic classes of *S. aureus* extracellular proteases (i.e. metallo-, serine and cysteine proteases [47]) exert a variety of virulence-related functions. These include tissue degradation [31, 32], effect on the host immune defense [33], interference with endogenous host enzymes [34] and the facilitation of bacterial dissemination [35, 36]. Here we show that degradation of SP-A by staphylococcal proteases may hamper bacterial clearance and facilitate the colonization of the human respiratory tract. SP-A interacts with a variety of bacteria and enhances pathogen uptake by phagocytic cells [5, 48, 49]. Human SP-A was shown to stimulate the serum-independent phagocytosis of *S. aureus* by alveolar macrophages and neutrophils [5, 23]. Furthermore, SP-A assists *S. aureus*

phagocytosis by monocytes via its interaction with the C1q receptor on these cells [11] and the SP-R210 and SR-A receptors on macrophages [23]. The SP-A concentration in the BALF of healthy individuals was found to be high, reaching a concentration of $>10 \mu\text{g}\cdot\text{ml}^{-1}$, thereby indicating even higher concentrations in surfactant fluid. Thus, in the current study SP-A was used at concentrations in the range of $7.5\text{--}25 \mu\text{g}\cdot\text{ml}^{-1}$. Similar concentrations were tested in previous reports [5, 50, 51].

Previous studies of the interaction between bacteria and the SP-A protein found that *P. aeruginosa* elastase can efficiently degrade SP-A. An analysis of BALF from lung transplant patients with cystic fibrosis revealed the presence of SP-A fragments resulting from protease-mediated degradation [51]. Careful analysis of our experimental data revealed that a similar phenomenon is observed for *S. aureus* with ScpA being the most efficient protease in SP-A molecule degradation. ScpA-mediated cleavage of SP-A resulted in a decrease or complete inhibition of SP-A biological activity, including the promotion of *S. aureus* phagocytosis by neutrophils, aggregation of Gram-negative bacteria and bacterial adherence to epithelial cells. Experimental analysis of complete BALF from human

lungs also showed that SP-A degradation by ScpA was not hampered by protease inhibitors or competing substrates that may be present in vivo. This experiment also showed that the amount of proteases secreted by clinical strains is sufficient to mediate cleavage. To further study the relevance of the process, an effort was made to determine whether staphopains are present in BALF from patients with staphylococcal respiratory tract infections. Immunoprecipitation followed by mass spectrometry SRM analysis failed to reveal the presence of ScpA in BALF samples (data not shown), which can be due to strain-specific variants of the enzyme primary structure. Unfortunately, no *S. aureus* strains were available to verify this possibility, which will be explored in future studies.

The results of in vivo studies investigating the role of ScpA in *S. aureus* infection are rather inconclusive [52]. Nevertheless, several in vitro studies showed that both staphopains are involved in the modulation of the infectious outcome [29, 37, 38, 53, 54]. The discrepancy between in vivo and in vitro models can be readily explained because in contrast to human serum, mouse serum inhibits staphopain activity. Both enzymes were shown to efficiently degrade human endogenous protease inhibitors (α -1-antitrypsin and α -1-antichymotrypsin) that are essential for the regulation of human serine protease activities [34, 55] and are important in protection of human lungs against uncontrolled proteolysis. Therefore, the deregulation of proteolytic homeostasis will most likely lead to excessive connective tissue degradation. This, together with the staphopain-mediated hampering of SP-A phagocytosis-promoting and -opsonizing functions may constitute ScpA as main virulence factors in the staphylococcal diseases of the lower respiratory tract. Overall, this study showed that the *S. aureus* protease ScpA functions as an important virulence factor that efficiently degrades SP-A, which is one of the key elements of lung innate immunity. Multiple models showed that degradation by ScpA results in the abolition of SP-A biological

activity. The function of SP-A protein is described in detail both in vivo and in vitro [2, 18–23], so it may be assumed that the process observed is of clinical relevance.

Clinical observations of SP-A levels during bacterial pneumonia are inconsistent, because some authors have reported a rapid decrease in SP-A levels [49] whereas others found increased protein levels during infection [56]. This discrepancy is probably attributable to the methodology applied by researchers, particularly whether the test employed detected the SP-A fragments or only the complete protein. In this context, more work is required to determine the in vivo role of SP-A proteolytic digestion in the development of *S. aureus*-related pneumonia.

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Disclosure Statement

The authors declare that they have no conflict of interests.

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