The cysteine protease ApdS from *Streptococcus suis* promotes evasion of innate immune defenses by cleaving the antimicrobial peptide cathelicidin LL-37

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ABSTRACT

*Streptococcus suis* is a globally distributed zoonotic pathogen associated with meningitis and septicemia in humans, posing a serious threat to public health. To successfully invade and disseminate within its host, this bacterium must overcome the innate immune system. The antimicrobial peptide LL-37 impedes invading pathogens by directly perforating bacterial membranes and stimulating the immune function of neutrophils, which are the major effector cells against *S. suis*. However, little is known about the biological relationship between *S. suis* and LL-37 and how this bacterium adapts to and evades LL-37–mediated immune responses. In the present study, using an array of approaches, including enzyme, chemotaxis, and cytokine assays; quantitative RT-PCR; and CD spectroscopy, we found that the cysteine protease ApdS from *S. suis* cleaves LL-37 and thereby plays a key role in the interaction between *S. suis* and human neutrophils. *S. suis* infection stimulated LL-37 production in human neutrophils, and *S. suis* exposure to LL-37 up-regulated ApdS protease expression in the bacterium. We observed that ApdS targets and rapidly cleaves LL-37, impairing its bactericidal activity against *S. suis*. We attributed this effect to the decreased helical content of the secondary structure in the truncated peptide. Moreover, ApdS rescued *S. suis* from killing by human neutrophils and neutrophil extracellular traps because LL-37 truncation attenuated neutrophil chemotaxis and inhibited the formation of extracellular traps and production of reactive oxygen species. Altogether, our findings reveal an immunosuppressive strategy of *S. suis* whereby the bacterium blunts the innate host defenses via ApdS protease–mediated LL-37 cleavage.

1. Introduction

Antimicrobial peptides, also known as host defense peptides, are key components of the innate immunity system, acting as the principal first line of defense against invading pathogens (1). As a sole member of the human cathelicidin family, the cationic peptide LL-37 is an extremely potent killer against different bacterial infections (1-3). This peptide can be strongly induced during injury and infection, and it is not only produced by epithelial cells, but also widely expressed in immune cells, such as neutrophils, monocytes, macrophages, and NK cells (4). LL-37 is derived from the carboxyl terminus of the precursor hCAP18 protein, which is stored in granules, and it is then released as a mature peptide in the extracellular environment to exercise its bactericidal effect (5). The characteristic positive charge allows LL-37 to associate with the negatively charged membranes of bacteria. During this interaction, LL-37 converts itself into an α helical conformation, which results in membrane penetration and bacterial lysis (6).

Of note, in addition to its outstanding bactericidal activity, LL-37 directly or indirectly exerts a wide range of immunomodulatory effects on a variety of immune cells, especially neutrophils (4,7). Neutrophils, the most abundant circulating leukocytes in the bloodstream, are considered to be the first cells of the innate immune system to migrate towards the site of infection (8). The modulation of LL-37 on neutrophils is the most diverse and critical, comprising chemotaxis, the production of reactive oxygen species (ROS), the formation of neutrophil
extracellular traps (NETs), and the induction of CXCL8 production et al. (9-12). Thus, as the main producer of LL-37, neutrophils both express and react to LL-37, and this feedback leads to high concentrations of LL-37 at the site of bacterial invasion. The burst of LL-37 further triggers a more effective immune response of neutrophils, which are capable of rapidly clearing the bacterial infection (13).

*Streptococcus suis* is an important zoonotic bacterial pathogen that constitutes a significant threat to human health (14). Since the first human case described in Denmark in 1968, over 1,600 human cases of *S. suis* infection occurred in more than 20 countries and regions, including Asia, North America, and Europe (15,16). Two severe outbreaks of human infections were reported in China in 1998 and 2005, resulting in 229 infections and 52 deaths (17,18). The most frequent clinical manifestations in humans are septicemia and meningitis. *S. suis* needs to survive well and proliferate in the bloodstream for maintaining a high level of bacteremia (14,19). During this process, it is vital that *S. suis* resists and evades from LL-37 and the neutrophil response, which are the first line of innate immune defenses. However, the *S. suis* -LL-37 interaction and the mechanism employed by the bacterium to thwart LL-37 and human neutrophils remain incompletely defined.

A previous study identified an aminopeptidase of *S. suis* that could cleave the arginine residues at the N-termini of the substrate and may have a potential contribution in virulence (20). This protease, designated as ApdS here, belongs to the family of cysteine proteases (20). In this study, we characterize the role of the cysteine protease ApdS in the interaction among *S. suis*, LL-37, and human neutrophils. Our data indicate that the cleavage of LL-37 by ApdS protease is one of the important mechanisms exploited by *S. suis* to subvert innate host defenses.

2. Results

2.1. *S. suis* infection stimulates cathelicidin LL-37 production in human neutrophils, while *S. suis* exposure to LL-37 upregulates the expression of the *apdS* gene

As a successful intruder into humans, *S. suis* manages to disarm the innate immune system and to disseminate to the organs through the bloodstream (14,19). To determine the role of the antimicrobial peptide LL-37 in the process of *S. suis* invasion, the LL-37 production in human neutrophils when exposed to *S. suis* wild-type (WT) strain 05ZYH33 was investigated using immunofluorescence microscopy with an antibody directed against LL-37. After the infection of neutrophils with *S. suis* at a multiplicity of infection (MOI) of 0.1, the production of hCAP-18/LL-37 was significantly increased and stored in secondary granules of neutrophils, as indicated by their colocalization with lactoferrin, which is the canonical marker of secondary granules (Fig. 1A and 1B). As shown in Fig. 1C, with an increasing number of *S. suis* at the time of infection, the expression of neutrophils with *S. suis* at a multiplicity of infection (MOI) of 0.1, the production of hCAP-18/LL-37 was significantly increased and stored in secondary granules of neutrophils, as indicated by their colocalization with lactoferrin, which is the canonical marker of secondary granules (Fig. 1A and 1B). As shown in Fig. 1C, with an increasing number of *S. suis* at the time of infection, the expression of hCAP-18 protein was upregulated, and the release of LL-37 was also increased. The intensity band ratio of hCAP-18/β-actin and LL-37/β-actin is shown in Fig. 1D and 1E. These results indicated that *S. suis* infection could quickly stimulate cathelicidin LL-37 production in human neutrophils.
underscoring the importance of LL-37 in the host defense against S. suis infection.

To examine the change of apdS gene expression during the response of S. suis to LL-37, the transcription level of the apdS gene was analyzed using quantitative real-time polymerase chain reactions (qRT-PCR). When the S. suis strain was treated in vitro with various sublethal concentrations (0.1, 0.2, and 0.4 μM) of LL-37, the growth curves and the number of surviving bacteria were similar to those of untreated bacteria (Fig. 1F and 1G), but the transcription levels of apdS were different (Fig. 1H). In the presence of increasing concentrations of LL-37, the expression of the apdS gene was upregulated in a dose-dependent manner. Moreover, with regards to the protein levels, LL-37 also stimulated the expression of the ApdS protein (Fig. 1I). The intensity band ratio of ApdS/GyrB is shown in Fig. 1J. These results suggest that apdS may aid S. suis in adapting to and resisting against LL-37 killing.

2.2. S. suis cysteine protease ApdS cleaves cathelicidin LL-37

ApdS protein consists of 607 amino acids (aa), containing an N-terminal signal peptide (1-25 aa), a C69 peptidase domain (26–474 aa), and a C-terminal LPSTG motif (574-578 aa, Fig. S1A). Multiple sequence alignment showed that the ApdS of S. suis exhibited similarity to the homologs of both Streptococcus pyogenes and Lactobacillus farriminis, with 45.8% and 24.6% sequence identity, respectively (Fig. S1B). To explore the structural features of ApdS, the three-dimensional structure (3D) of ApdS was simulated using that of Lactobacillus farriminis C69-family cysteine peptidase (PDB: 5iau) as a template. Automated molecular modeling of the 3D structure generated a homology model consisting of 13 α-helices and 16 β-sheets. The N-terminal Cys26 is predicted to be the catalytic site, which is located in the middle of the 3D structure (Fig. 2A).

To explore the proteolytic activity of the ApdS protease, S. suis ApdS and its catalytic site-mutated protein ApdS(C26A) were overexpressed in the E. coli BL21 (DE3) strain and the recombinant His6-tag fusion proteins were purified using Ni2+-affinity chromatography (Fig. 2B). Since the C69 family of cysteine protease has N-terminal aminopeptidase activity, the cleavage specificity of ApdS was determined on four chromogenic peptidase substrates. Two (L-Leu-pNa and Gly-pNa) were rapidly hydrolyzed, whereas no obvious activity was detected against L-Pro-pNa and Gly-Pro-pNa. The $K_m$ and $K_{cat}$ values for the Michaelis–Menten kinetics of ApdS were 0.222 mM and 202 S$^{-1}$ on L-Leu-pNa, 0.298 mM and 104 S$^{-1}$ on Gly-pNa (Fig. 2C).

To determine the ability of ApdS to cleave LL-37, we incubated LL-37 in the presence or absence of ApdS or ApdS(C26A) and analyzed the samples by mass spectrometry. A reduction in mass of LL-37 after ApdS treatment was found that is equivalent to the loss of its respective N-terminal LLG amino acid (Fig. 2D). The cleavage product was designated as LLG-cleaved peptide (abbreviated as LLGCP). However, there was no reduction in the mass of LL-37 treated with ApdS(C26A) (Fig. 2D), proving that Cys26 is the catalytic site of ApdS protease.

2.3. ApdS-dependent cleavage of LL-37 impairs its bactericidal activity against S. suis

To determine whether the ApdS protease has a role in assisting S. suis
resisting against LL-37 killing, an apdS gene deleted mutant strain (ΔapdS) and its complemented strain (CΔapdS) were constructed and confirmed by PCR and DNA sequencing (Fig. S2). The results of qRT-PCR showed that the transcription levels of the downstream genes of apdS in WT and ΔapdS were similar, confirming that the deletion of apdS was nonpolar (Fig. S3A). Furthermore, ApdS transcription and protein expression in ΔapdS were virtually undetectable and restored in CΔapdS (Fig. S3B and S3C), and the growth of the three strains was similar (Fig. S3D). The survivability of the WT, ΔapdS, and CΔapdS strains upon exposure to the LL-37 was evaluated with a bactericidal assay. As shown in Fig. 3A, the ΔapdS mutant was significantly more sensitive than the WT strain to LL-37 over a concentration range of 1–4 μM. Additionally, the sensitivity of ΔapdS to LL-37 was increased with the extension of incubation time (Fig. 3B). Trans-complementation with apdS expressed on a pSET2 plasmid restored resistance to LL-37.

Subsequently, we compared the bactericidal ability of LL-37 and LLGCP on S. suis. Since the WT strain can express the ApdS protease to cleave LL-37, the bactericidal ability of LL-37 and LLGCP was evaluated in the ΔapdS mutant, thus excluding the interference of the ApdS protease. As shown in Fig. 3C, LLGCP had a weaker bactericidal activity compared to LL-37, as indicated by the number of surviving bacteria (CFUs/ml) following incubation in the presence of each peptide. To compare the effect of these peptides in the bacterial membrane integrity, the permeability of the ΔapdS mutant after LL-37 and LLGCP treatment was confirmed using the SYTOX green dye. SYTOX green is a cationic cyanine dye that can only penetrate compromised membranes, and then binds to the nucleic acids of bacteria causing a large increase in fluorescence intensity. SYTOX green fluorescence was measured after the addition of each peptide to S. suis cells suspended in PBS buffer. Treatment with LL-37 showed accelerating membrane permeabilization, as indicated by a concentration-dependent increase in fluorescence intensity (Fig. 3D). Although LLGCP treatment also showed the SYTOX green influx, the degree of permeabilization was significantly lower than that of LL-37, suggesting their different bactericidal ability against S. suis (Fig. 3D). These findings indicate that the ApdS protease is crucial for protecting S. suis from the bactericidal activity of LL-37.

2.4. The cleavage of LL-37 influences its α-helical conformation

The permeabilizing effect of the antimicrobial peptide on bacterial membranes is frequently associated with the presence of a specific secondary structure (21). Previous studies have indicated that the interaction of LL-37 with the bacterial membrane components should result in conformational changes of this peptide (22). To investigate the impact of the cleavage of LL-37 by ApdS on the α-helical secondary structure, the structural features of LL-37 and LLGCP in different environments were examined, and the Circular dichroism (CD) spectra are illustrated in Fig. 4. In water, the spectra for both LL-37 and LLGCP exhibited a typical random-coil conformation (Fig. 4A). However, the spectra for LL-37 in PBS (pH 7.4) exhibited an α-helical structure, which is consistent with the results reported by
Zelezetsky et al. (23). The spectrum for LLGCP also showed a helical character, but with only 23.4% helical content compared to the 37.1% helical content of LL-37 (Fig. 4B). Moreover, 50% trifluoroethanol (TFE) has a strong helix-promoting property and has been frequently used as a membrane-like environment. In this condition, the spectra for both LL-37 and LLGCP showed a greater helical character, 53.3% and 43.1%, respectively (Fig. 4C). In addition, 30 mM SDS has been frequently used for mimicking the negatively charged bacterial membrane environment. In this condition, the helical content of LL-37 increased to 63.0%, while LLGCP had a helical content of 38.5% (Fig. 4D). These results revealed that the helical contents of LL-37 are significantly higher than those of LLGCP in the PBS and membrane-mimicking environments. Compared to LLGCP, the more stable helical character of LL-37 when interacting with the bacterial membrane may contribute to its superior antimicrobial activity.

2.5. ApdS aids S. suis to resist the killing by neutrophils and NETs

Being produced by phagocytes, mainly neutrophils and macrophages, cathelicidin LL-37 significantly promotes the immune defenses against invasive infections (4,7). It has been found that LL-37 is produced at high concentration of 0.2–1.5 μM in most bodily fluids, but at infection sites the concentration can increase more than 6–10 times (24-26,28). For instance, LL-37 is present in the bronchoalveolar lavage fluid (BALF) at 3.3 μM in healthy individuals but can increase up to 13.1 μM and 25.5 μM in patients with pulmonary and systemic infections (26,27). To investigate the role of the ApdS protease in the evasion of innate immune responses by S. suis, the ability of the WT, ΔapdS, and ΔapdS strains to survive in human blood, macrophages, neutrophils, and NETs was examined. The concentrations of LL-37 in these different environments were determined (Fig. 5A, 5B, 5C and 5D). Compared to the WT strain, the viability of ΔapdS after exposure to human blood was markedly reduced, and ΔapdS rescued the survival defect of the ΔapdS mutant in the blood (Fig. 5E). In addition, compared to the WT strain, the ΔapdS mutant became more susceptible to killing by THP-1 macrophages when LL-37 expression was induced with vitamin D (Fig. 5F). The survival ability of the WT, ΔapdS, and ΔapdS strains was further evaluated in bactericidal assays using human neutrophils and NETs (which were induced with phorbol 12-myristate 13-acetate (PMA) from human neutrophils). The results showed that the WT strain was more resistant to human neutrophil killing than the ΔapdS mutant, and it also survived better within NETs (Fig. 5G and 5H). Taken together, in accordance with the role of ApdS in evasion from LL-37 killing, ApdS also protects S. suis from killing by LL-37-producing phagocytes, which shows the crucial role of ApdS in S. suis resisting and escaping from innate immune-mediated killing.

2.6. ApdS-dependent cleavage of LL-37 attenuates its chemotactic ability to attract human neutrophils

How could ApdS mediate neutrophil evasion? An attractive hypothesis is that ApdS-dependent cleavage of LL-37 not only impairs its bactericidal activity, but also destroys its immunomodulatory properties for neutrophils. Previous studies have found that LL-37 enhanced lysosome
formation in phagocytes (10). Thus, we first assessed the ability of LL-37 and LLGCP to induce the lysosome formation in neutrophils. Unexpectedly, LL-37 and LLGCP induced lysosome formation to similar extents (Fig. S4). These data indicated that ApdS-dependent cleavage of LL-37 did not damage the immunomodulatory properties of LL-37 for lysosome formation.

A notable feature of LL-37 is that it has excellent chemotactic properties for neutrophil migration towards sites of infection (4,9,27). To examine whether the cleavage of LL-37 by ApdS affects its chemotactic ability, the neutrophils were pre-labeled with CellTracker and their migration promoted by LL-37 and LLGCP was monitored using a transwell assay. The results indicate that LL-37 stimulated remarkable chemotaxis of neutrophils in a dose-dependent manner up to a concentration of 2 μM. However, in the presence of LLGCP, a significant reduction in neutrophil migration was observed compared with that of LL-37 induction (Fig. 6A). It was also observed that LL-37 could promote chemotaxis through the induction of chemokine CXCL8 production (12). In line with the results of chemotaxis, compared to the untreated group, both LL-37 and LLGCP stimulated the CXCL8 production in a dose-dependent manner. However, LLGCP exhibited a reduced CXCL8 immunostimulatory effect compared with the potent ability of LL-37 (Fig. 6B). Collectively, these data demonstrated that ApdS protease could impair the chemotactic property of LL-37 required for the migration of human neutrophils to the local site of S. suis infection, which can prevent the subsequent functional activation of human neutrophil for phagocytosis.

2.7. ApdS-dependent cleavage of LL-37 inhibits its ability in promoting NETs formation and ROS production

An interesting feature of LL-37 is that it can facilitate NETs formation, which is one of the fundamental innate immune defense mechanisms (11). To investigate whether ApdS has an effect on the ability of LL-37 in NETs formation, LL-37 and LLGCP were used to stimulate human blood-derived neutrophils. The NETs were observed using immunofluorescence microscopy with an antibody directed against DNA/histone 1 complexes. As shown in Fig.7A, neutrophils alone without peptide incubation did not produce the NET structure. As a positive control, PMA could effectively activate neutrophils to form a characteristic NET structure. In addition, extensive web-like structures into the extracellular space were released when neutrophils were incubated with 8 μM of LL-37. Neutrophils stimulated with the same concentration of LLGCP also released extracellular DNA-containing fibers, but the area of NET structure was significantly reduced compared with that of LL-37. However, when neutrophils were treated with 2μM of peptides, neither LL-37 or LLGCP could trigger NETs formation (Fig. 7B). To further define whether the cleavage of LL-37 by ApdS affects ROS production, a fluorescent ROS dye was used to evaluate LL-37 and LLGCP for intracellular ROS production. As shown in Fig. 7C, LL-37 induced significantly more ROS production at the concentrations of 4–8 μM than LLGCP, suggesting that the cleavage of LL-37 by ApdS affects its ability for ROS production. Taken together, relatively high concentration of LL-37 could significantly stimulate NETs formation, which is
consistent with the results of a previous study (11), and ApdS protease could attenuate the effect of LL-37 on NETs formation and ROS production.

3. Discussion

The host innate immune system plays a critical role in the control of invading microbial pathogens. A key feature of this defense is the production of antimicrobial peptides. The only human cathelicidin, LL-37, shows a broad-spectrum bactericidal activity against both Gram-negative and Gram-positive bacteria, various viruses, and fungi (1). The concentration of LL-37 can increase more than 6–10 times in patients during infection (24-26,28), which means that there is a surge of LL-37 surrounding the activated neutrophils at a site of infection. Similarly, in the model of the human blood-cerebrospinal fluid barrier, S. suis infection significantly induced the transcript expression of LL-37 (29).

During the process of infection, S. suis is always exposed to the killing effect of LL-37, and how this bacterium deals with LL-37 has remained so far enigmatic. Here, we demonstrated for the first time that S. suis targets and cleaves LL-37 through the activity of the ApdS protease, resulting in impaired bactericidal activity towards S. suis. Additionally, the ApdS-dependent cleavage of LL-37 also impaired its immunomodulatory properties for human neutrophils. Since neutrophils are the major effector cells in the innate immune defense against S. suis (29,30), ApdS protease is expected to be a novel immune evasion factor involved in S. suis pathogenesis.

S. suis commonly causes acute clinical symptoms in humans (31), suggesting that this bacterium is able to quickly and effectively thwart the innate immune response before the activation of the adaptive immune response. Our study demonstrated that LL-37, as the first line of the innate immune system, could be stimulated within few minutes after S. suis infection. Intriguingly, the increased concentration of LL-37 further up-regulated the transcript expression of the apdS gene, implying that S. suis could efficiently adapt to the host environment in response to LL-37, and it may employ the ApdS protease to resist the killing by LL-37. Notably, although the action of LL-37 is fast and only takes a few minutes to kill bacteria (32), the proteolytic activity of ApdS protease is even more rapid and the cleavage of LL-37 is completed within merely a few seconds, further illustrating the importance of ApdS for S. suis in combating the early immune response.

Our results showed that the cleavage of LL-37 by ApdS protease results in the removal of three amino acids (LLG) from LL-37, and this truncated peptide (LLGCP) has been found to be less active than LL-37 against S. suis. Like other cationic peptides, the mechanism exploited by LL-37 to kill bacterial cells is the perforation of their cytoplasmic membranes. The initial LL-37-membrane interactions are mediated through electrostatic properties that the net positive charge facilitates LL-37 to interact with the net negative charge of bacterial surfaces, containing lipopolysaccharides or lipoteichoic acids (1). Thus, the changes in net charge of a peptide can influence its electrostatic affinity for bacterial cell surfaces, which further influences its bactericidal activity. At neutral pH, LL-37 is highly charged with net charge of +6, but after cleavage by ApdS, the net charge of LLGCP remains +6, suggesting that the
reduced bactericidal activity of LLGCP is not related to the net charge of this peptide. In addition to charge, the structural conformation of peptide is also a major factor for its bactericidal activity (33,34). LL-37 presents a disordered conformation in aqueous solutions, but forms an α-helical conformation in the presence of physiological salt concentration (22). Our data of the CD spectra revealed that LLGCP experienced the same structural transition as LL-37 from disordered to helical structure. In the membrane-mimetic environments, this conformation is further stabilized as indicated by an increased helical content. However, in either physiological environment or membrane-mimetic environments, the helical content of LLGCP is always lower than that of LL-37. These results adequately explain why the bactericidal activity of LL-37 is reduced after the cleavage by ApdS. In line with this, it has been reported that the extent of helicity correlates with the antibacterial activity of LL-37 (22,23). The truncated peptide of LL-37, which contains four residue deletions at the N terminus, has been showed to be less helical, leading to a decrease in bactericidal activity when compared with that of LL-37 (22). Taken together, with the help of ApdS protease, S. suis avoids and resists the LL-37-mediated killing, and the decreased bactericidal activity of the cleaved peptide LLGCP is attributed to the diminished helical content of its secondary structure.

Until now, many pathogens have been found to successfully evade the LL-37-mediated killing by producing surface-associated or secreted proteases. A metalloprotease of Staphylococcus aureus, Aureolysin, inactivated the antimicrobial peptide LL-37 by cleaving the C-terminal peptide bonds and promoted the bacterial survival within the LL-37-rich environment of macrophage phagolysosomes (35). Similarly, a metalloprotease of Tannerella forsythia, karilysin, also cleaved LL-37 and significantly reduced its bactericidal activity (36). Rapala-Kozik et al. had found that the pathogenic candida albicans could effectively use aspartic proteases to destroy the antimicrobial and immunomodulatory properties of LL-37, thus enabling the pathogen to survive and propagate (37). In Streptococcus pyogenes, the proteolytic inactivation of LL-37 by the cysteine protease SpeB was also reported (38). Additionally, pathogenic bacteria also have other LL-37 resistance mechanisms. The M1 surface protein from group A Streptococcus (GAS) was found to contribute to the LL-37 resistance of this bacterium through sequestering and neutralizing LL-37 (39). In addition, efflux pumps are used by many bacterial pathogens to resist LL-37 by extruding it from the cell membrane site of action to the extracellular environment. The MefE/Mel efflux pump contributed to LL-37 resistance in S. pneumoniae (40), while the Pmt ABC transporter of S. aureus was shown to be essential for defending the bacteria from killing by LL-37 (41).

The antimicrobial peptide resistance mechanisms of S. suis have remained unclear until now. Only the study of Tim et al reported that knockout of the dltA gene in S. suis gives rise to the absence of lipoteichoic acid D-alanylation, leading to increased susceptibility to the bacterium-derived peptide colistin, polymyxin B, and the frog-derived peptide magainin II (42). The lipoteichoic acid D-alanylation has been found to reduce the
net negative charge of lipoteichoic acids and relieve the electrostatic attraction between cationic antimicrobial peptide and the bacterial cell envelope (43). Although not described in the study of Tim et al., we speculate that D-alanylation of lipoteichoic acids also blunts the access of LL-37 to the surface of S. suis. Nonetheless, the lavish amounts of LL-37 released by neutrophils still surround S. suis and further attract more and more neutrophils towards the infection site to engulf the bacteria. Interestingly, our study revealed a novel ApdS protease-driven mechanism exploited by S. suis to impede the chemotaxis of neutrophils. The cleavage of LL-37 by the ApdS protease not only directly impairs its chemotactic activity, but it also indirectly hinders the migration through the down-regulation of the CXCL8 production, which is a key chemokine for neutrophils.

Of note, LL-37 is also able to facilitate the formation of NETs, which is a novel anti-microbial defense strategy against pathogenic bacteria (11,44,45). Although the underlying mechanism of LL-37 for NETs formation is still unknown, we found that a high concentration of LL-37 indeed promotes the formation of NETs, consistent with previous research (11). In fact, the concentrations of LL-37 released at infection sites through neutrophil degranulation are much higher than the concentration in our study used for NETs formation, and NETs are likely to be found at places with a high microbial burden (26,27,45). Thus, we surmise that the neutrophils can estimate the quantity of bacteria by sensing the concentration of LL-37 stimulated by bacteria. Once bacteria reach the quantity that LL-37 can no longer control, neutrophils may initiate NETs formation through an unknown mechanism to imprison the bacteria. A recent study also demonstrated that LL-37 could act as a second signal to induce inflammasome activation in airway epithelial cells and promote an inflammasome-mediated altruistic cell death of Pseudomonas aeruginosa infected epithelial cells (46). It is clearly that LL-37 act as a “fire alarm” to enhance rapid escalation of inflammatory responses to an uncontrolled bacterial infection (46). Furthermore, different LL-37-derived truncated peptides have been demonstrated to exhibit significant differences in the NETs formation (11). Our study found that the cleavage of LL-37 by ApdS protease weakened the ability of LL-37 for NETs formation, and the apdS deletion increased the sensitivity of S. suis to NETs killing. Since it has been reported that the wild-type S. suis and different nuclease mutants did not show significant differences in bacterial survival within NETs (29), it seems that the nucleases cannot effectively help S. suis to escape from NETs. Thus, NETs may be protected by LL-37 against bacterial nucleases, and the survival of S. suis within NETs may result from its ability to resist the killing by LL-37.

At present, antibiotic resistance of pathogenic bacteria to conventional drugs is already a global health concern. To cope with this grim situation, antimicrobial peptides are being considered as important candidates for developing novel antibiotics (47), and many of them now undergo preclinical or clinical development, including LL-37 (48). Additionally, designing and improving the antimicrobial peptides for the optimal therapeutic application is an ongoing task (49). Amino acids Arg and Lys, both of which carry positive charges at neutral pH, are
commonly used to design antimicrobial peptides (50). We found that the ApdS protease of S. suis can also cleave Arg and Lys from the N-terminus of the peptide (data not shown), thus reminding us that these amino acids need to be arranged to the suitable location or modified when designing antimicrobial peptides for therapy of the S. suis infection.

In summary, the present study provides evidence of a novel strategy of immune evasion by which S. suis manipulates LL-37 and neutrophils, thwarting the innate immunity. The key event is the targeting and cleaving of the LL-37 to prejudice its bactericidal activity and immunomodulatory properties for human neutrophils. These findings also inspire the consideration for the current design strategies of antimicrobial peptides needs to be improved for boosting its therapeutic application to avoid being damaged by microorganisms, as well as to enhance its immunomodulatory function.

4. Experimental procedures

4.1. Bacterial strains and growth conditions

The bacterial strains and plasmids used for this study are described in Table 1. The S. suis WT strain used in this study was serotype 2 strain 05ZYH33 (GenBanK: CP000407.1), which isolated from a dead patient in Sichuan Province, China in 2005. The S. suis strains were cultured in a Todd-Hewitt broth (THB, Difco Laboratories, Detroit, MI, USA) medium or plated on THB agar. For the preparation of competent cells, S. suis strains were grown in THB supplemented with 2% yeast extract (THY). Escherichia coli strains were cultured in Luria-Bertani (LB, Difco Laboratories) medium or plated on LB agar. When necessary, antibiotics were added to the plate or broth at the following concentrations: chloramphenicol (Sigma, St. Louis, MO), 5 μg/ml for S. suis, 10 μg/ml for E. coli; spectinomycin (Sigma), 100 μg/ml for S. suis, 50 μg/ml for E. coli; erythromycin (Sigma), 5 μg/ml for S. suis, 180 μg/ml for E. coli; and ampicillin (Sigma), 100 μg/ml for E. coli. All of the strains were routinely grown at 37°C.

4.2. Cell and culture conditions

THP-1 macrophages (human leukemic cell line) were grown in RPMI 1640 Medium (Gibco). Human neutrophils were isolated from freshly collected whole-blood samples (EDTA-treated) obtained from healthy volunteers using the PolymorphPrep system (Axis Shield) as previously described (39) and resuspended in RPMI 1640 medium. For isolation of human neutrophils, written informed consents were obtained from the donors in accordance with the declaration of Helsinki (2013) of the World Medical Association. The medical ethics committee of the First Affiliated Hospital of Dalian Medical University approved the use of human blood for this study (No. LCKY2016-35(X)).

4.3. LL-37 production analysis

Neutrophils were seeded into the glass-bottom culture dish at a density of 1 × 10^5 cells per dish, then infected with S. suis at a MOI of 0.1, 1, and 5, and incubated at 37°C for 40 min. After washing with PBS, the cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100. Nonspecific binding was blocked by incubation with 3% bovine serum albumin in PBS for 60 min. Then cells were incubated with rabbit-anti-LL-37 (Anaspec) for 60 min at 37°C. After washing three times, the cells were stained with Alexa Fluor 488-conjugated goat anti-rabbit IgG,
and co-stained with 4'6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St Louis, MO, USA) to visualize nuclei. Fluorescence images were obtained using the LSM 800 confocal laser scanning microscope (Carl Zeiss, Germany). Quantification of area stained for LL-37 was performed using ImageJ software (National Institute of Health, Bethesda, USA).

4.4. In vitro growth assays

The S. suis strain 05ZYH33 was grown to mid-logarithmic phase in THB medium, and then diluted to an optical density at 600 nm (OD$_{600}$) of 0.1 in 4 ml of THB medium supplemented with LL-37 (GL peptide Inc., purity> 98%, concentration range 0–0.8 μM). Bacterial growth at 37°C was monitored by measuring the OD$_{600}$ values at an interval of 1 h using an Eppendorf BioPhotometer (Eppendorf, Germany).

4.5. qRT-PCR

S. suis strain 05ZYH33 was grown in 4 ml of THB medium supplemented with LL-37 (concentration range 0–0.4 μM). The cultures were harvested at OD$_{600}$ 1.0 by centrifugation at 8,000 g at 4°C for 10 min. Total RNA was extracted from each sample using the RNeasy Mini kit (Qiagen), and complementary DNAs (cDNAs) was synthesized using the PrimeScript RT reagent kit (TaKaRa, Japan). Transcript amounts of apdS were determined in a Stratagene Mx3000P system (Agilent Technologies, Germany) by quantification relative to the housekeeping gene gyrB (Table 2) as described previously (51). qRT-PCR were performed in a MicroAmp Optical 96-well reaction plate using SYBR Green I (TakaRa) according to the manufacturer’s instructions. All of the reactions were performed in triplicate with three independent biological replicates. Relative expression levels were determined using the comparative threshold cycle (ΔΔCt) method to calculate the fold change in gene expression (52).

4.6. Bioinformatics analysis

The signal peptide cleavage site was predicted using SignalP 4.0 program (53). Multiple protein sequences were aligned using ClustalW software (54), and presented by ESPript program (55). The active catalytic site was predicted was using MEROPS database (56). Protein structure homology modeling was constructed by SWISS-MODEL (57) and presented by PyMOL (58).

4.7. Protein expression and purification

The coding sequence of S. suis ApdS residues C26-L574 was PCR-amplified from the 05ZYH33 genomic DNA using primers apdSF/R (Table 2). The DNA fragments and pET22b vector (Novagen) were digested with NdeI and XhoI (Takara) for ligation to each other. The construction of recombinant plasmid pET22b-apdS was confirmed by sequencing and transformed into competent E. coli BL21 (DE3) cells. The protein expression was induced for 15 h at 16°C by the addition of 0.5 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) in LB broth containing 50 μg/ml ampicillin. The His6- tag fusion proteins were purified by Ni Sepharose™ 6 Fast Flow column (GE Healthcare, United States). Protein concentrations were determined using a bicinchoninic acid protein assay kit (Beyotime, China).

For site-directed mutagenesis, the DNA segment including the mutation of Cys26 to Ala was amplified from the plasmid pET22b-apdS using primers C26AF and apdSR (Table 2). The digested PCR product was ligated with NdeI/XhoI-digested pET22b, and the construct was confirmed by DNA
sequencing. The mutated protein ApdS(C26A) was expressed and purified as described above.

4.8. Enzymatic assay

Amidolytic activities of ApdS protease was measured using the substrate L-Leu-pNa, Gly-pNa, L-Pro-pNa and Gly-Pro-pNa (Sigma, 1 mM final concentration) in PBS buffer (pH 7.4) at 37°C. Assays were performed in 100 μl on 96-well plates using a thermostated microplate reader, and the release of p-nitroaniline was measured at 405 nm (BioTek, USA). Kinetic values were measured using L-Leu-pNa and Gly-pNa at various concentrations ranging from 31 μM to 2 mM, with a fixed enzyme concentration at 30 nM, in PBS buffer (pH 7.4) at 37°C. V_max and K_m values were obtained through hyperbolic regression analysis.

For proteolytic degradation of LL-37, 2 μM of LL-37 was incubated with 20 nM of ApdS and ApdS(C26A) in 100 μl of PBS buffer. Control reaction lacked ApdS to obtain uncleaved LL-37. Reactions were incubated at 37°C for 20 min and submitted for mass spectrometry analysis using an AB SCIEX 5800 MALDI-TOF/TOF mass spectrometer (AB Sciex, Canada).

4.9. Construction of gene deletion mutant

The primers used for the construction of the deletion mutant ΔapdS are listed in Table 2. The DNA segments corresponding to the upstream and downstream regions of the apdS gene were amplified from the genomic DNA of WT strain 05ZYH33 using primers UF/U_R and DF/DR carrying PstI/XhoI and BamHI/EcoRI restriction enzyme sites, respectively. The Spc^r gene cassette was amplified from plasmid pSET2 (59) with primers spcF/R carrying XhoI/BamHI restriction enzyme sites. The three digested DNA fragments were ligated by incubation with T4 DNA ligase for 6 h at 22°C, and PCR was carried out with primers UF and DR. The DNA fragment was digested with PstI/EcoRI and cloned into the plasmid pSET6S to produce the plasmid pSET6SΔapdS. To obtain the isogenic mutant ΔapdS, the pSET6SΔapdS was then electroporated into the WT strain 05ZYH33 as described previously (60). After homologous recombination, the apdS gene was replaced by the Spc^r cassette and the S. suis ΔapdS mutant was verified by sequencing and PCR assays with a series of specific primers listed in Table 2. The transcription levels of the downstream genes of apdS in WT and ΔapdS were analyzed using qRT-PCR with specific primers (Table 2).

4.10. Complementation of the S. suis ΔapdS mutant

The 1,974 bp DNA fragment containing the entire apdS open reading frame (ORF) and 150 bp of the upstream promoter were amplified with the primers CF/CR (Table 2). The 1,185bp DNA fragment containing the entire erm open reading frame (ORF) and the upstream promoter were amplified with the primers ErmF/R. Both amplicons were subsequently cloned into E. coli -S. suis shuttle plasmid pSET2 (51), yielding the recombinant plasmid pSET2-erm-apdS. This plasmid was confirmed by DNA sequencing (Comate Bioscience Co., Ltd.) and transformed into the ΔapdS mutant for trans complementation. The complemented strain CΔapdS was selected on THB agar supplemented with 5 μg/ml of erythromycin, and verified by PCR and DNA sequencing using the primers CF/CR and ErmF/R. The transcription and protein expression levels of ApdS in WT, ΔapdS, and CΔapdS
were analyzed using qRT-PCR with specific primers (Table 2) and western blot.

4.11. Western blot
Protein samples fractionated by SDS-PAGE were transferred onto nitrocellulose membranes (GE Healthcare). Membranes blocked with 5% bovine serum albumin in PBST were incubated overnight at 4°C with an appropriately diluted primary antibody in PBST containing 2% BSA. After incubation with DyLight 800 goat anti-rabbit IgG (H+L), blots were visualized by using an Odyssey infrared imaging system (Li-Cor BioSciences, Lincoln, NE).

4.12. LL-37 bactericidal assays
LL-37 bactericidal assays were performed as described previously (61). The S. suis WT strain 05ZYH33, ΔapdS mutant, and the complemented strain CΔsapA were grown in THB medium at 37°C to OD_600 0.8. Each strain from the broth cultures was harvested and diluted in PBS (pH 7.4) to a concentration of 1 × 10^4 CFU/ml. The wells of a sterile, polystyrene 96-well microtiter plate (Costar 3599, U.S.A.) were filled with 90 μl of the various concentration (0–4 μM) of LL-37. Ten microliters of the bacterial suspension were added to each well, and the plate was incubated for 0–60 min at 37°C. Bacteria incubated with PBS served as controls. Serial dilutions of the bacteria were plated on THB agar for CFU counting.

4.13. SYTOX green uptake assay
The S. suis ΔapdS mutant was grown to OD_600 0.8. The strain from the broth culture was washed three times and diluted to 1 × 10^5 CFU/ml in PBS (pH 7.4). The bacteria were incubated with SYTOX green (Invitrogen) for 15 min in the dark, after which they were mixed with the appropriate concentration (0–4 μM) of LL-37 and LLGCP (GL peptide Inc., purity > 98%) for 30 min. Fluorescence was measured using an EnSpire Multilabel Reader (PerkinElmer, Singapore), with excitation at 485 nm and emission at 520 nm.

4.14. Circular dichroism spectroscopy
The secondary structures of LL-37 and LLGCP in different environments were measured using CD spectroscopy. The LL-37 and LLGCP peptides were respectively analyzed at 8 μM concentration in deionized water, PBS (pH 7.4), 50% TFE, and 30mM SDS. The CD spectra were recorded as the average of three scans at 1-nm intervals between 198–250 nm using a CD spectrometer (Chirascan, Applied Photophysics, England) with a 1-cm path length quartz cuvette. The helical content of these peptides in different environments was analyzed using the BeStSel tool (62).

4.15. Phagocytes bactericidal assays
The bactericidal assays were used to compare the growth of WT, ΔapdS mutant, and CΔapdS in human blood, THP-1 macrophages, and a neutrophil killing assay as previously described (39,63). Briefly, diluted cultures of strains (50 μL, 5 × 10^4 CFU/mL) were combined with 450 μL of fresh human blood, and the mixtures were incubated for 0-1.5 h at 37°C. The viable cell counts were determined by plating diluted samples onto THB agar. The percent of live bacteria was subsequently calculated using the CFU of bacteria in the original inoculum as 100%.

For THP-1 killing, THP-1 cells were treated with 50 nM vitamin D (active form, 1,25-dihydroxyvitamin D3) for 36 h and then infected with S. suis strains at MOI of 1:50 for 3 h at 37°C under 5% CO₂. In neutrophil killing assays, neutrophils were
infected with S. suis strains at MOI of 1:50 for 3 h. For NETs killing, neutrophils were stimulated with 25 nM PMA (Sigma) 4 h pre-infection. Serial dilutions of the bacteria were plated on THB agar. Colonies were counted, and the percentage of surviving bacteria was calculated using the CFU of bacteria incubated in RPMI 1640 as 100%.

4.16. Dot blot

To determine the concentrations of LL-37 in human plasma, THP-1 macrophages, neutrophils, and NETs during S. suis infection, dot blot analysis was performed as previously described (26,64). Briefly, after treatment, the cells were pelleted by centrifugation at 800g for 20 minutes. 3 μl of each supernatant sample were dotted onto a nitrocellulose membrane, immunolabeled with rabbit-anti-LL-37 (Anaspec), followed by DyLight 800 goat anti-rabbit IgG (H+L). Signal intensity of each blot was quantified using an Odyssey infrared imaging system (Li-Cor BioSciences, Lincoln, NE). Standard peptide concentrations were used to calculate the concentrations in the biologic samples.

4.17. Lysosome formation analysis

Neutrophils were seeded into the glass-bottom culture dish at a density of 5 × 10⁴ cells per dish, then incubated with 0–2 μM of LL-37 and LLGCP for 4 h at 37°C under 5% CO₂. The procedures of fixation, permeabilization, and blocking were the same as described in the experiment for LL-37 production analysis. The cells were incubated with mouse anti-LAMP1 (Abcam) for 60 min at 37°C, then stained with Alexa Fluor 647-conjugated goat anti-mouse IgG and DAPI. Fluorescence images were obtained using the LSM 800 confocal laser scanning microscope (Carl Zeiss, Germany). Quantification of area stained for lysosome was performed using ImageJ software.

4.18. Neutrophil chemotaxis assay

Neutrophil migration was evaluated by the 24-well microchemotaxis chamber technique as described previously (37,65). Briefly, Neutrophils were labeled with CellTracker Green (Invitrogen) and washed three times. Then 1 × 10⁶ cells were seeded in the upper chambers of 5-μm-pore Transwell inserts (Corning Life Sciences). Chemotactic samples of the indicated concentrations of LL-37 and LLGCP were placed in RPMI 1640 medium in the lower chambers, with the 5μM fMLP (Sigma) used as a positive control. Following incubation for 40 min at 37°C under 5% CO₂, the neutrophil migration was monitored by fluorescence microscopy (Zeiss) and the percentage of chemotactic cells was calculated using the number of migrating cells in the lower the chamber of positive control as 100%.

4.19. CXCL8 production assay

Neutrophils (1 × 10⁶ cells/well) were suspended with 500 ul of RPMI medium in 24-well plates. The cells were incubated with various concentration (0–8 μM) of the LL-37 and LLGCP for 6 h at 37°C under 5% CO₂. Supernatants were collected and placed in a sterile 96-well plate for ELISA. CXCL8 production was determined by an enzyme-linked immunosorbent assay kit (Sigma) according to the manufacturer’s instructions.

4.20. NETs formation assay

Neutrophils were seeded into the glass-bottom culture dish at a density of 2 × 10⁵ cells per dish pre-coated with poly-L-lysine, then incubated with 0–8 μM of LL-37 and LLGCP for 5 h at 37°C under 5% CO₂. As a positive control, 25
nM of PMA was used to trigger the NETs formation. The procedure of fixation, permeabilization, and blocking were the same as described in experiment for LL-37 production analysis. The cells were incubated with mouse anti-DNA/histone H1 (Merck Millipore) for 60 min at 37°C, then stained with Alexa Fluor 488-conjugated goat anti-mouse IgG and DAPI. Fluorescence images were obtained using the LSM 800 confocal laser scanning microscope (Carl Zeiss, Germany). Quantification of area stained for NETs was performed using ImageJ software.

4.21. ROS production assay

The level of cellular ROS was measured using the fluorescence probe 2’,7’-dichlorofluorescein diacetate (DCFDA, Sigma) as described previously (10). Briefly, human neutrophil cells (2 × 10^5) were treated with 0–8 μM of LL-37 and LLGCP at 37°C under 5% CO₂. After treatment, the cells were washed with PBS three times and stained with 5μM DCFDA for 15 min in the dark. Cells were washed three times with PBS and the fluorescence intensity of the cells was read using an EnVision Multilabel Reader (PerkinElmer, UK), with an excitation wavelength of 485 nm and an emission wavelength of 520 nm to represent ROS activity.

4.22. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA, U.S.A.). Data were analyzed by performing an unpaired two-tailed Student’s t-test or one-way ANOVA. P-values less than 0.05 were considered statistically significant.
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Table 1
Characteristics of bacterial strains and plasmids used in this study

| Strains or plasmids | Characteristics or sequence | Source or reference |
|---------------------|----------------------------|---------------------|
| **Strains**         |                            |                     |
| *E. coli* DH5α      | *E. coli* strain used for cloning | Invitrogen         |
| *E. coli* BL21(DE3) | *E. coli* strain used for expression | Invitrogen         |
| *S. suis* 05ZYH33   | Virulent Chinese serotype 2 strain isolated from a dead patient in an outbreak in Sichuan, China, 2005 | Accession no. CP000407.1 |
| *S. suis* Δ*apdS*   | Gene *apdS* knockout mutant of *S. suis* strain 05ZYH33, Spc<sup>e</sup> | This work          |
| *S. suis* CΔsapA    | The complemented strain of *S. suis* Δ*apdS* containing the *apdS* ORF and 150 bp of the upstream region, Spc<sup>e</sup>Em<sup>r</sup> | This work          |
| **Plasmids**        |                            |                     |
| pET22b              | Expression vector, Amp<sup>r</sup> | Invitrogen         |
| pET22b-Δ*apdS*      | Recombinant expression plasmid to produce His<sub>6</sub>-fused ApdS protein | This work          |
| pET22b-Δ*apdS*(C26A)| Recombinant expression plasmid to produce His<sub>6</sub>-fused ApdS(C26A) protein | This work          |
| pSET2               | *E. coli*-*S. suis* shuttle expression vector, Spc<sup>e</sup> | Accession no. AB042430.1 |
| pSET6S              | Temperature-sensitive *E. coli*-*S. suis* shuttle vector for gene replacement in *S. suis*, Cm<sup>r</sup> | Accession no. AB055652.1 |
| pSET6SΔ*apdS*       | pSET6S derivative carrying a deletion of *apdS*, Spc<sup>e</sup>Cm<sup>r</sup> | This work          |
| pSET2-erm-Δ*apdS*   | pSET2 with a PCR-derived insert containing the *apdS* gene and erm gene, Spc<sup>e</sup> Em<sup>r</sup> | This work          |
Table 2
Primers used in this study

| Primer     | Sequences (5’ – 3’)                                                                 | Source or reference |
|------------|------------------------------------------------------------------------------------|---------------------|
| apdSF      | 5’-TGCCATATGTGTTCCAGGCTTTATTATGGG-3’, upstream primer with internal NdeI site (underlined) | This work           |
| apdSR      | 5’-GGCACTCGAGAAGACTTGAATTACCTTTTTATTGGG-3’, downstream primer with internal XhoI site (underlined) | This work           |
| C26AF      | 5’-TGCCATATGGCCTCAAGCCTTTATTATGGG-3’, upstream primer with internal NdeI site (underlined) for site-directed mutagenesis of Cys26 to Ala on ApdS protein | This work           |
| RTapdSF    | 5’-CGTTCTCGGACATACGCTGG-3’, for qRT-PCR                                             | This work           |
| RTapdSR    | 5’-TGCCGCTTTGGTCATC-CG-3’, for qRT-PCR                                              | This work           |
| RTgyrBF    | 5’-GGACCCTGGGTGCTTAACAGA-3’, for qRT-PCR                                            | This work           |
| RTgyrBR    | 5’-AGGTGGTACCCATGAGCAAG-3’, for qRT-PCR                                             | This work           |
| RTcopAF    | 5’-CGGTTGAAGAAGCGGTGGG-3’, for qRT-PCR                                              | This work           |
| RTcopAR    | 5’-AGAGCACAAGGAAGCAGTGC-3’, for qRT-PCR                                             | This work           |
| RTtpxF     | 5’-ACGGTTGATACCGTTTCACGC-3’, for qRT-PCR                                            | This work           |
| RTtpxR     | 5’-ACAGCCTCCAAAGGCCGATC-3’, for qRT-PCR                                             | This work           |
| UF         | 5’-AACTCAGGTCATCCACGATTTGACGCA-3’, upstream primer with internal Pstl site (underlined) | This work           |
| UR         | 5’-CAGTCTCCAGAGCATAGACTTCCCTTTCTGATACTC-3’, downstream primer with internal XhoI site (underlined) | This work           |
| DF         | 5’-CAGTGATCTCTAATAGAGCTAGTAAAGAATAGGTTG-3’, upstream primer with internal BamHI site (underlined) | This work           |
| DR         | 5’-CGGAATTCGAGCAAAGCTGGTAAACGGAAG-3’, downstream primer with internal EcoRI site (underlined) | This work           |
| spcF       | 5’-CAGTCTCGAGTACAGGATTTACCGTAC-3’, upstream primer with internal XhoI site (underlined) | This work           |
| spcR       | 5’-CAGTGATCTCTTCTAATCAGTAATTTACCGTAC-3’, downstream primer with internal BamHI site (underlined) | This work           |
| CheckF     | 5’-GAGTTGAAAGGATATCTCCCTTTTTGACGAC-3’, upstream primer for identification of ΔapdS mutant | This work           |
| CheckR     | 5’-GCTCTATCTTCTCAAACCTCAAAG-3’, downstream primer for identification of ΔapdS mutant | This work           |
| UoutF      | 5’-TTGTAAGACGAAAGGAGAGAATAATG-3’, upstream primer for identification of ΔapdS mutant | This work           |
| UoutR      | 5’-ACAGATCGCTTGAGATAGGGCC-3’, downstream primer for identification of ΔapdS mutant | This work           |
| DoutF      | 5’-TTGCGGGGAATGCGATGCGC-3’, upstream primer for identification of ΔsapA mutant      | This work           |
| DoutR      | 5’-CATTTCGCAAGACCTGAGCC-3’, downstream primer for identification of ΔsapA mutant | This work           |
| Primer | Sequence | Description |
|--------|----------|-------------|
| gdhF   | 5'-GCAGCGTATCTGTCAAAACG-3', upstream primer for identification of *S. suis* strain | This work |
| gdhR   | 5'-CCATGGACAGATAAGATGG-3', downstream primer for identification of *S. suis* strain | |
| CF     | 5'-GATCCTGCAGCAATGTAGGTGCAAAATGGTA-3', upstream primer with internal PstI site (underlined) | This work |
| CR     | 5'-CGGGATCCCTTTTTACTGGATTTTTTTTG-3', downstream primer with internal BamHI site (underlined) | |
| ErmF   | 5'-GTTTGGATCCCCGAAGCAAACCTAAAGAGTGTGTTG-3', upstream primer with internal BamHI site (underlined) | This work |
| ErmR   | 5'-CTGAATTCAAATTCCCCGTCAGGCGCT-3', downstream primer with internal EcoRI site (underlined) | |
Figure 1. *S. suis* infection stimulates the production of LL-37 in human neutrophils, and the increased levels of LL-37 upregulate the expression of the ApdS protein.

(A) Microscopy analysis of LL-37 production in human neutrophils. Neutrophils were infected with the *S. suis* WT strain 05ZYH33 at a MOI of 0.1 for 40 min. The production of hCAP-18/LL-37 was significantly increased and stored in secondary storage.
granules of neutrophils. Nuclei are shown in blue, hCAP-18/LL-37 in green, and lactoferrin in red. Mock, uninfected neutrophils. Scale bars = 5 μm. (B) Changes in LL-37 production were evaluated by the quantification of the green area using ImageJ. Values represent the mean ± SD, n = 6 donors, *** P < 0.001. (C) S. suis infection increased the expression level of hCAP-18/LL-37. Neutrophils were infected with S. suis (MOI = 0.1, 1, and 5), and cells and supernatants were respectively collected after centrifugation and subjected to western blot with anti-hCAP-18/LL-37 antibody. β-actin levels served as the loading control. The intensity band ratios of (D) hCAP-18/β-actin and (E) LL-37/β-actin were calculated and compared. Values represent the mean ± SD from three experiments, * P < 0.05, ** P < 0.01, *** P < 0.001. (F) The growth curves of S. suis WT strain when exposed to increasing concentrations of LL-37. Bacteria was first grown in THB for 8 h and then diluted to OD600 0.1, and incubated with different concentrations of LL-37 at 37°C. Points indicate the mean values, and error bars indicate standard deviations. (G) The survival characteristics of S. suis WT strain when exposed to increasing concentrations of LL-37. After 5 h incubation, each sample was plated on THB agar for identification of viable bacteria. Each experiment was performed in triplicate. Values represent the mean ± SD, * P < 0.05, *** P < 0.001, n.s. = not significant. (H) Transcriptional alteration of the apdS gene was examined using qRT-PCR in WT strain when exposed to increasing, yet sublethal concentrations of LL-37. Values represent the mean ± SD from three experiments performed in triplicate, *** P < 0.001. (I) The expression of ApdS protein was examined using western blot analysis with mouse-anti-ApdS in WT strain when exposed to increasing concentrations of LL-37. GyrB levels served as the loading control. (J) The intensity band ratios of ApdS/GyrB was calculated and compared. Values represent the mean ± SD from three experiments, ** P < 0.01, *** P < 0.001.
Figure 2. *S. suis* ApdS protease targets and cleaves LL-37.

(A) 3D model of ApdS. Cartoon showing the α-helix, β-sheet, and random coil in red, yellow, and green. The catalytic active site (Cys26) is shown as spheres in blue. Protein structure homology modeling was constructed by SWISS-MODEL using the 3D structure of *L. farciminis* C69-family cysteine peptidase (PDB: 5iau) as a template, and presented by PyMOL. (B) SDS-polyacrylamide gel showing the purified ApdS and...
Apds(C26A) expressed by *E. coli*. (C) Proteolytic activities of ApdS with L-Leu-pNa and Gly-pNa used as a substrate. For kinetic values determination, final substrate concentrations ranging from 31 μM to 2 mM were used. The $V_{\text{max}}$ and $K_m$ values were determined from the Michaelis-Menten equation using the GraphPad Prism software. (D) ApdS cleaves LL-37. LL-37 was incubated alone (top panel), with ApdS (middle panel) or with ApdS(C26A) (bottom panel) at 37°C and then analyzed by mass spectrometry.
Figure 3. ApdS protease protects S. suis from the bactericidal activity of LL-37.

(A) S. suis WT, ΔapdS, and CΔapdS strains were incubated with increasing concentrations of LL-37 for 40 min, and (B) 2 μM of LL-37 for increasing incubation time. Each sample was plated on THB agar for identification of viable bacteria. Values represent the mean ± SD from three experiments performed in duplicate, ** \( P < 0.01 \), *** \( P < 0.001 \).

(C) Bactericidal activities of LL-37 and LLGCP on S. suis ΔapdS. The bacteria were exposed to increasing concentrations of LL-37 and LLGCP. Each sample was plated on THB agar for identification of viable bacteria. Values represent the mean ± SD from three experiments performed in duplicate, *** \( P < 0.001 \).

(D) Membrane permeabilization of LL-37 and LLGCP on S. suis ΔapdS. The bacteria loaded with SYTOX green were exposed to the indicated concentrations of LL-37 and LLGCP, and the fluorescence (excitation at 485 nm and emission at 520 nm) were monitored. Values represent the mean ± SD from three experiments performed in duplicate, ** \( P < 0.01 \), *** \( P < 0.001 \).
Figure 4. The cleavage of LL-37 by ApdS protease influences its α-helical content.

The secondary structures of LL-37 and LLGCP were determined by CD spectra at the concentration of 8 μM in different environments. (A) deionized water, (B) PBS, pH 7.4, (C) 50% TFE, (D) 30mM SDS. The percentages of helical content were calculated using the BeStSel tool.
Figure 5. ApdS protease aids *S. suis* to evade the killing by human neutrophils and NETs.

LL-37 concentrations in different environments. (A) Freshly collected human whole-blood was infected with/without 1 × 10^5 CFU/mL of *S. suis* for 5 h. (B) THP-1 macrophages (5 × 10^7 cells) were treated with/without 50 nM vitamin D (active form,
1,25-dihydroxyvitamin D3) for 36 h. (C) Neutrophils (1 × 10^7 cells) were infected with/without *S. suis* (MOI=5) for 6 h. (D) Neutrophils (1 × 10^7 cells) treated with/without 25 nM PMA for 5 h. After centrifugation, the supernatant of each sample was collected and subjected to dot blot analysis. Standard peptide concentrations were used to calculate the concentrations in the biologic samples. Values represent the mean ± SD, n = 6 donors, ** *P* < 0.01, *** *P* < 0.001, n.s. = not significant. Survivals of *S. suis* WT, ΔapdS, and CΔapdS strains in (E) human blood, (F) THP-1 macrophages pre-treated with/without 50 nM vitamin D (active 1,25D_3 form) for 36 h, (G) human neutrophils, (H) NETs pre-induced with 25 nM PMA from human neutrophils. The percentage of surviving bacteria was calculated using the CFU of bacteria incubated in RPMI 1640 as 100%. Values represent the mean ± SD, n = 6 donors, * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001.
Figure 6. The cleavage of LL-37 by ApdS protease decreases its chemotactic ability to attract human neutrophils.

(A) Human neutrophils were seeded in the upper chamber of a 5 μm Transwell, migration was measured stimulated with various concentrations of the LL-37 and LLGCP in the lower chamber. 5 μM of fMLP was used as positive control. Neutrophil chemotaxis was calculated using the number of migrating neutrophils attracted by positive control fMLP as 100%. Values represent the mean ± SD, n = 6 donors, *P < 0.05, ** P < 0.01. (B) CXCL8 production by human neutrophils in response to LL-37 and LLGCP. Cells were incubated with various concentrations of peptides for 6 h at 37°C. Supernatants were collected and CXCL8 levels were measured by ELISA. Values represent the mean ± SD, n = 6 donors, * P < 0.05, ** P < 0.01.
Figure 7. The cleavage of LL-37 by ApdS protease impairs its ability to promote NETs formation and ROS production.

(A) Microscopic visualization of NETs formation stimulated by 8 μM of LL-37 and LLGCP for 5 h at 37°C. NETs stimulated from human neutrophils were stained for DNA/histone H1 in green, nuclei in blue. Mock, unstimulated neutrophils. 25 nM of PMA was used as positive control. Scale bars = 20 μm. (B) Changes in NETs formation stimulated by the indicated concentrations of LL-37 and LLGCP were evaluated by the quantification of the green area using ImageJ. Values represent the mean ± SD, n = 6 donors, ** P < 0.01, *** P < 0.001, n.s. = not significant. (C) ROS production stimulated by the indicated concentrations of LL-37 and LLGCP was measured using the fluorescence probe DCFDA. Values represent the mean ± SD, n = 6 donors, * P < 0.05, ** P < 0.01, n.s. = not significant.
The cysteine protease ApdS from *Streptococcus suis* promotes evasion of innate immune defenses by cleaving the antimicrobial peptide cathelicidin LL-37

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