The Pro-Inflammatory Effect of Staphylokinase Contributes to Community-Associated Staphylococcus Aureus Pneumonia

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Article

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The pro-inflammatory effect of Staphylokinase contributes to community-associated Staphylococcus aureus pneumonia

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Abstract

Pneumonia caused by community-associated *Staphylococcus aureus* (CA-SA) has high morbidity and mortality. Pathogens can express a variety of virulence factors to promote infection. The game between the host immune system and pathogens determines the degree of infection and tissue damage, but its mechanism has not been well explored. In this study, we found that staphylokinase (SAK) is highly expressed in CA-SA lineage ST398 and promotes lung infection in both wild-type and cathelicidins-related antimicrobial peptide CRAMP knockout (*CRAMP*^{-/-}) mice. Furthermore, our results showed that SAK can activate the host's innate immune inflammatory response. Importantly, SAK can promote the activation of NLRP3 inflammasome by increasing the production of ROS. This may be one of the important mechanisms of SAK aggravating CA-SA pneumonia after excluding its function of promoting fibrinolysis and neutralizing CRAMP. Our results emphasize the importance of controlling inflammation in acute lung infections, and also provides new insights into the pathogenesis of highly virulent CA-SA.

Keywords

Community-associated *Staphylococcus aureus*, Staphylokinase, Pneumonia, Excessive inflammation, NLRP3 inflammasome

Introduction

Staphylococcus aureus (*S. aureus*) is one of the most important pathogens and can cause a wide variety of infections^{1,2}. During the past decade, *S. aureus* has become an important cause of community acquired pneumonia³⁻⁵. In contrast with hospital-associated *S. aureus* (HA-SA), which usually infects patients with impaired immunity, community-associated *S. aureus* (CA-SA) infections can occur in other healthy individuals, suggesting that these bacterial strains have greater virulence⁶.

High expression of virulence genes that promote invasive infections plays an important role in the pulmonary pathology associated with CA-SA related pneumonia^{5,7,8}. However, excessive local inflammation and tissue damage can also impede bacterial clearance⁹. Mouse pneumonia models lacking innate immune signal components showed significantly improved outcomes when infected by *S. aureus*^{10,11}. The NLRP3 inflammasome is essential for the host's immune defense against bacterial, fungal and viral infections¹². But, when NLRP3 is dysregulated or overactivated, the activated caspase-1 promotes the maturation of IL-1 β and IL-18, facilitates the recruitment of neutrophils and participates in the inflammatory process of acute lung injury damage^{9,13-15}.

The ST398 clone was previously considered to be livestock-associated, but more and more epidemiological studies have shown that ST398 isolates spread in community populations are usually not related to animal infections¹⁶. The isolation rate of ST398 continues to increase, and it has become one of the most prevalent CA-SA clones in Shanghai, China¹⁶⁻¹⁸. We have reported the effects of high expression levels of

virulence genes such as the ESAT-6 secretion system on the pathogenicity of ST398^{19,20}. In addition, prophage also plays an important role in bacterial pathogenicity and evolution^{21,22}. The gene encoding staphylokinase (SAK) is located on Prophage 3²³ and it is conserved in human adapted *S. aureus* (HO-SA)¹⁶. SAK was originally found to promote the activation of plasminogen and exert its fibrinolytic function in specific hosts^{24,25}. Moreover, studies have shown that SAK can bind to α -defensins²⁶, human cathelicidin LL-37 and mouse cathelicidins-related antimicrobial peptide (CRAMP)²⁷ to regulate fibrinolysis and evade innate immunity defenses. While the research on SAK has often focused on its fibrinolytic function, the role of SAK in the pathogenesis of *S. aureus* remains insufficient and controversial.

In the present study, our results showed increased expression of SAK in ST398 and ST59 isolates, pointing to a potential role of SAK in the virulence of CA-SA. Moreover, we demonstrated a significant role of SAK in the acute ST398 infectious pneumonia model, which is mainly achieved by activating innate immune signals, especially NLRP3 inflammasome-related pathways.

Results

***sak* is conserved in HO-SA and is highly expressed in CA-SA**

Prophage 3 is considered as a potential molecular marker to distinguish livestock-adapted *S. aureus* (LA-SA) from HO-SA²⁸. In the present study, we detected the carrying rate of *sak* in the predominant HA-SA (ST5, ST239), CA-SA (ST59, ST188, ST398) and LA-SA (ST188, ST398, ST97, ST520) isolates of China. The results showed that 96-100% of HO-SA carried the *sak* gene, and there was no significant

difference between HA-SA and CA-SA, while the detection rate of *sak* in LA-SA was only 0-28% (Figure 1A). Among these LA-SA, ST398 and ST188, which carry a higher proportion of *sak* than ST97 and ST520, can be isolated from both humans and animals. These results further confirmed that the carrier rate of *sak* is related to human infection.

Although the *sak* gene is conserved in HO-SA, we found that the expression level of *sak* in CA-SA was significantly higher than that in HA-SA (Figure 1B). It has been shown that the increased expression of pathogenicity-related genes is responsible for the high virulence of CA-SA^{19,20}. We speculate that the high expression of SAK may also play an important role in promoting the pathogenesis of CA-SA. In addition, we used a chromogenic assay to measure the level of SAK secreted by bacteria in cultures with different incubation times. The results showed that the high SAK secretion level of CA-SA reached its peak in the logarithmic growth phase and remained stable (Figure 1C).

SAK's effect on biofilm formation is plasminogen-dependent

SAK can promote the activation of human plasminogen and exert its fibrinolytic function. However, SAK can only activate plasminogen in some specific hosts, including humans, rabbits, and sheep, but not mice²⁴. Studies in mice expressing human plasminogen showed that SAK can inhibit the formation of biofilms²⁴. Biofilms are involved in antibiotic resistance of bacteria and host immune defenses²⁹. But the highly virulent *S. aureus* often causes invasive infections¹⁹ through high expression of virulence-related genes, and their biofilm formation ability is usually weaker than HA-SA (Figure 1D).

By detecting the activation effect of SAK on plasminogen, we confirmed the successful construction of *sak* gene knockout strain and the *sak* complementary strain (Supplementary Figure 1A). We found that *sak* deletion did not affect the growth of ST398 and the formation of biofilm in TSB medium (Figure 1E, Supplementary Figure 1B). Furthermore, the addition of SAK protein did not inhibit the formation of biofilms, but after adding human plasma, significant differences were observed between the wild-type and the *sak* mutant isolates (Figure 1E). This indicates that the ability of SAK to inhibit biofilm formation is achieved primarily by promoting fibrinolysis in specific hosts, and these results are consistent with the research of Kwiecinski et al²⁴. Although CA-SA with high SAK expression showed weak biofilm formation ability (Figure 1B-D), we speculated that this is more likely due to the high expression level of the important virulence regulator system, such as accessory gene regulator (Agr) system (Supplementary Figure 2A). High expression of the *agr* locus can inhibit the formation of biofilms³⁰, which is consistent with our results (Supplementary Figure 2B). The Agr system positively regulates a variety of virulence-related genes, and the expression of *sak* is also regulated by it, but the knockout of *sak* gene does not affect the expression of Agr system. (Supplementary Figure 2C-D). Therefore, we speculated that the inhibitory effect of SAK on biofilm formation depends on fibrinolysis.

SAK of ST398 can increase the severity of acute lung infection in mice

It is reported that SAK can directly bind to mouse CRAMP, helping to promote fibrinolysis and evasion of the host's innate immunity²⁷, so we used both wild-type and *CRAMP*^{-/-} mice for study. The lung tissues of ST398-infected wild-type mice showed

obvious congestion and edema (Figure 2A). HE staining of the lung tissues showed that the alveolar structure of mice was destroyed after infection with the ST398 strain, with infiltration of a large number of inflammatory cells (Figure 2A). Immunohistochemistry analysis of lung tissues also proved that ST398-infected mice had more macrophage infiltration (Figure 2B). Furthermore, the wet weight of the lungs and the colony-forming unit (CFU) counts in lung tissues of ST398-infected mice increased significantly (Figure 2C, D). Interestingly, similar results can be seen in *CRAMP*^{-/-} mice (Figure 2E-G). After infection with the *sak* mutant strain, both wild-type mice and *CRAMP*^{-/-} mice had milder lung pathological changes. Therefore, we speculated that SAK can increase the severity of acute lung infection in mice, and the effect of combining with CRAMP is not the main mechanism of SAK's pathogenicity.

The pro-inflammatory effect of SAK in CA-SA pneumonia

In order to explore the mechanism of SAK in the process of *S. aureus* infection, we analyzed the RNA sequencing data of lung tissues from wild type and *sak* gene mutant ST398 infected mice (C57BL/6 wild-type). A total of 580 genes were differentially expressed between the two groups. In addition, the results of GSEA analysis identified that genes related to cytokine response, such as interferon- γ (IFN- γ), interferon- α (IFN- α), interleukin-6 (IL-6), interleukin-1 (IL-1), and complement-related genes, and NLRP3 inflammasome-related genes were enriched in the lung tissues of wild-type infected mice (Figure 3A). Moreover, we detected 23 cytokines in the serum of mice, and found that mice infected with wild-type ST398 had higher levels of serum cytokines (Figure 3B).

In the host's confrontation with pathogens, excessive inflammation can cause tissue damage and poor prognosis. The activation of NLRP3 has been shown to promote cytokine production and cell pyrolysis. We further compared the expression of toll-like receptors, NF- κ B and other NLRP3 inflammasome activation-related genes in the two groups. These molecules are almost all highly expressed in the lung tissues of mice infected with wild-type ST398 (Figure 3C). In addition, we detected the expression of IL-1 β , IFN- α , *nlrp3* and *caspase-1* in lung tissues by qRT-PCR, and the results were consistent with RNA sequencing (Figure 3D-G). Combined with the pathological changes in mouse lung tissue, we speculate that the lung tissue of mice infected with wild-type ST398 is in a more active state of inflammatory response than that of *sak* gene mutant ST398 infected mice.

SAK promotes the activation of NLRP3 inflammasome

We incubated BMDM of mice with bacterial culture supernatant or SAK protein and extracted the mRNA of the cells. Gene expression was detected by qRT-PCR after reverse transcription, and the results showed that SAK can effectively promote the expression of innate immune inflammation-related factors, such as cytokines (IL-17, TNF- α , IL-1 β , IL-8) and NLRP3 inflammasome-related genes (Figure 4A-F). It should be noted that although the expression level of *caspase-1* and IL-1 β genes increased 2-fold after SAK protein treatment, we did not observe a significant increase in cleaved caspase-1 by western blot (Supplementary Figure 3). Therefore, whether the SAK protein can directly activate the NLRP3 inflammasome remains to be further studied. Moreover, what we can demonstrate is that in the presence of effective NLRP3

activators (such as LPS or *S. aureus* culture filtrates), SAK can further promote the maturation of caspase-1 and IL-1 β , and the difference between the wild-type strain and the *sak* mutant strain can also prove this point (Figure 4G, H). However, when the BMDM of *caspase-1*^{-/-} mice were incubated with bacterial supernatant or SAK protein, SAK could no longer promote the expression and maturation of IL-1 β (Figure 4I, J). This indicates that the activation of the NLRP3 inflammasome plays an important role in the process of SAK promoting the maturation and release of IL-1 β .

Inhibition of the NLRP3 inflammasome can reduce lung tissue damage caused by ST398

We constructed a lethal mouse model of pneumonia to visually show the effect of SAK on the pathogenicity of ST398. The results showed that *sak* gene knockout can improve the survival rate of ST398-infected mice (Figure 5A). Correspondingly, HE staining of lung tissue sections of mice infected with wild-type ST398 showed a high degree of inflammatory cell infiltration, and immunohistochemistry analysis showed high levels of IL-1 β (Figure 5B). In addition, the NLRP3 inflammasome can be inhibited by intraperitoneal injection of MCC950 (NLRP3 inhibitor) to mice. When mice were given NLRP3 inhibitors, their mortality decreased, and there was no significant difference in the survival rate of mice infected with wild-type ST398 and *sak* mutant strains (Figure 5C). This indicates that NLRP3 inflammasome activation plays an important role in SAK's promotion of the pathogenicity of ST398. Furthermore, the inhibitory effect of MCC950 on the NLRP3 inflammasome was confirmed by immunohistochemical staining of IL-1 β , and HE staining of lung tissue sections showed

that inhibiting NLRP3 inflammasomes can reduce the recruitment of inflammatory cells and weaken the inflammatory damage caused by infection (Figure 5D).

SAK exerts a pro-inflammatory effect on THP1 cells, and can promote the production of ROS

Furthermore, we measured the cytokines released by human THP1 cells (human monocyte derived cell line) after stimulation with bacterial culture supernatant. Similar to the results in mouse models, SAK can also stimulate the release of inflammatory factors (IL-1 β , TNF- α , IL-17A) in THP1 cells (Figure 6A-C). This indicates that the activation of the host inflammatory response by SAK is also applicable to human *S. aureus* infections.

The NLRP3 inflammasome can be activated by a variety of stimuli, such as ion flux, production of ROS, and lysosomal damage¹². In order to explore the mechanism by which SAK enhances the inflammatory response and promotes the activation of NLRP3 inflammasomes, we used potassium channel blocker glibenclamide, NADPH oxidase inhibitor apocynin, lysosome membrane stabilizer dexamethasone, and ROS scavenger N-acetyl-L-cysteine (NAC) to treat THP1 cells. The results showed that the addition of apocynin, dexamethasone and NAC could significantly reduce the expression of IL-1 β gene in the SAK treatment group, and eliminate the expression difference between ST398 wild type and the *sak* mutant group (Figure 6D). In addition, only apocynin and NAC could inhibit the promoting effect of SAK on IL-1 β secretion (Figure 6E). Apparently, the inhibition of K⁺ efflux by glibenclamide does not change the effect of *S. aureus* culture supernatant on IL-1 β secretion. Moreover, although the

secretion of IL-1 β can be significantly reduced by adding dexamethasone, there are still statistical differences between the wild type strain and the *sak* mutant strain (Figure 6E). Therefore, we consider that destroying lysosomes is not the main mechanism by which SAK promotes the pathogenicity of *S. aureus*. Since NADPH oxidase is proven to be an important source of ROS, we speculate that ROS production may play an important role in SAK's promotion of inflammation. Furthermore, by detecting the content of intracellular ROS, we found that SAK can significantly promote the generation of ROS, which can also be inhibited by NAC (Figure 6F).

Discussion

In our present study, we demonstrate that the expression level of SAK in CA-SA is significantly higher than that of HA-SA, and it plays an important role in acute necrotizing pneumonia by promoting the generation of ROS and activating NLRP3 inflammasomes. This may be a new mechanism for SAK to promote infection in addition to promoting fibrinolysis and neutralizing antimicrobial peptides. We have summarized the main functions of SAK and presented them in Figure 7.

Previous reports have shown that SAK can promote the activation of plasminogen in specific hosts, thus inhibiting the formation of biofilms and facilitating the spread of bacteria²⁴. The effect of SAK on biofilm formation is human plasminogen-dependent^{24,25}, which is consistent with our results. It is worth noting that although the SAK highly expressed CA-SA biofilm formation ability is weak, there is no evidence that SAK can directly inhibit the formation of *S. aureus* biofilm. It has been found that the increased expression of virulence factors controlled by Agr is responsible for the

high pathogenicity of ST398, and we speculate that the weak biofilm formation ability of CA-SA is more likely due to the high expression of the Agr system. In addition, the expression of SAK is also positively regulated by the Agr system. Furthermore, it has been proven that SAK can directly bind to murine CRAMP and promote fibrinolysis, while also playing a role in evading the host's innate immunity²⁷. In this study, our data indicate that increased expression of *sak* may promote lung infection of ST398 isolates in both wild-type and *CRAMP*^{-/-} mice. Since the effect of SAK on human plasminogen is species-specific, we speculate that promoting fibrinolysis and evading host innate immunity may not be the main mechanism of SAK aggravating pneumonia in ST398-infected mice.

Bacterial infection of different tissues and organs will cause different pathological consequences. In acute lung infections caused by high virulence CA-SA, uncontrolled excessive and long-term inflammation induced by virulence factors can lead to cell death and tissue damage^{9,31,32}, thereby hindering bacterial clearance^{11,33}. Inflammation is a double-edged sword for the body to resist microbial infections. Studies have shown that the loss of alveolar macrophages and immune regulatory dysfunction caused by caspase-1 dependent pyroptosis can amplify the pathological consequences of infection^{13,32}. It is worth noting that RNA sequencing analysis of mouse lung tissue showed that SAK can promote the activation of the innate immune inflammatory response. Our results demonstrated that SAK can significantly promote the activation of NLRP3 inflammasomes in mouse BMDM. Although we are still not sure whether SAK can directly induce the maturation of caspase-1, in the presence of an effective

NLRP3 inflammasome activator, SAK can further promote the maturation of caspase-1 and the release of IL-1 β . Furthermore, the application of NLRP3 inflammasome inhibitors can significantly reduce the mortality of mice, and SAK no longer exhibits the effect of promoting infection. This may preliminarily explain the role of NLRP3 inflammasome activation in SAK's promotion of pathogenesis. In addition, SAK can also promote the release of cytokines from human THP1 cells, which indicates that the activation of host inflammatory response by SAK is also suitable for human infections.

Various stimuli including bacterial toxins can activate the NLRP3 inflammasome, but no direct interaction between NLRP3 and these stimuli has been observed³⁴. It seems that they may activate NLRP3 by inducing various cell signal transduction events, including K⁺ efflux, ROS production and lysosomal damage¹². In this study, we found that SAK can promote the release of ROS in THP1 cells, and scavenging ROS or inhibiting NADPH oxidase can eliminate the influence of SAK on IL-1 β release. It is worth noting that in the RNA sequencing study of mouse lung tissue, we found that SAK can promote the expression of the NLRP3 inflammasome upstream molecules, such as NF- κ B and toll-like receptors, which may be another possible means of NLRP3 activation.

In previous studies, researchers tended to pay more attention to the promoting effect of SAK on fibrinolysis. Our research found that SAK is also an effective pro-inflammatory factor for acute infections caused by CA-SA. This enriches our understanding of the function of SAK, and at the same time, we emphasize that in the pneumonia caused by CA-SA, the control of inflammation is as important as the anti-

infection strategy. However, how SAK promotes the production of ROS and the mechanism of its pro-inflammatory effect needs to be confirmed by further studies.

In conclusion, this study preliminarily explored the pro-inflammatory role and mechanism of SAK in acute *S. aureus* pneumonia. Our results emphasize the importance of controlling inflammation in acute lung infections, and also provides new ideas for the pathogenic mechanism of high virulence CA-SA.

Methods

Bacterial isolates

S. aureus strains were isolated from adult patients of Renji Hospital affiliated with Shanghai Jiaotong University. The concentration of antibiotics used when constructing gene knockout strains is as follows: ampicillin, 100µg/ml; chloramphenicol, 10µg/ml. Please see the Supplementary data-1 for more details.

Measurement of SAK secretion by *S. aureus*

Overnight cultures (12-15 hours) were diluted 1:100 into fresh TSB and incubated at 37°C with shaking at 200 rpm. Bacterial suspension at different time points was centrifuged (4000 rpm for 10 minutes at 4°C) and the supernatant was collected after filtration. Human plasminogen (Roche) was mixed with supernatant and incubated at 37°C for 30 minutes, then a plasmin-specific chromogenic substrate S-2251 (3 mmol/L, Chromogenix) was added and incubation was continued at 37°C for 60 minutes. The amount of SAK secreted by the strains was read with a micro enzyme-linked immunosorbent assay (ELISA) autoreader (BioRad) at 405 nm.

Semiquantitative biofilm assay

Cells were fixed by Bouin's fixative after incubation at 37°C for 24 hours in TSB

medium with 0.5% glucose or 10% plasma. Heparin anticoagulated human plasma was provided voluntarily by healthy subjects. Mouse plasma came from the experimental C57BL/6 mice. Both human and mouse plasma were incubated at 56°C for 30 minutes before use to inactivate complement. Wells were washed by sterile phosphate buffered saline (PBS) after fixative for 1 hours, and then the biofilm was stained by crystal violet. The floating stain was washed off with running water and the ability of biofilm formation was reflected by the absorbance at 570 nm (Micro ELISA autoreader, BioRad).

Lung infection model in mice

C57BL/6 wild-type or *CRAMP*^{-/-} (Jackson Laboratory) female mice (7-8 weeks old) were used for the lung infection model. *S. aureus* strains were grown to mid-logarithmic phase, washed once and then resuspended with sterile PBS at 5×10⁶ CFU/μl. Mice were anesthetized with 2,2,2-tribromoethanol (3.75-5mg /25g), and then 40 μl inoculum was pipetted into the nares of the anesthetized mice slowly. For the fatal pneumonia model, 50 mg/kg MCC950 sodium (NLRP3 inflammasome inhibitor, Selleck) or sterile PBS was injected intraperitoneally into mice two hours before infection by 10⁹ CFU (2×10⁷ CFU/μl, 50μl) *S. aureus*. Please see the Supplementary data-1 for more details.

Cell culture

Mouse bone marrow derived macrophages (BMDM) were isolated from bone marrow and differentiated for 7 days in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin (100U/ml) and streptomycin (0.1mg/ml) and 20 ng/ml human M-CSF (R&D Systems). THP1 cells (human monocytic leukemia cell

line) were cultured in RPMI 1640 medium with FBS and penicillin and streptomycin. Phorbol 12-myristate 13-acetate (100ng/ml, 24 hours) was used in differentiation of THP1 into macrophages. The additives or inhibitors used in in vitro experiments are as follows: purified SAK (0.5µg/ml), lipopolysaccharide (LPS, 0.2µg/ml) / ATP (5mM), potassium channel blocker glibenclamide (10µM, Sangon Biotech), NADPH oxidase inhibitor apocynin (100µM, Selleck), lysosome membrane stabilizer dexamethasone (100µM, Sangon Biotech), and reactive oxygen species (ROS) scavenger N-acetyl-L-cysteine (NAC, 10µM, Sigma).

Quantitative reverse-transcription (qRT) PCR

Total RNA of *S. aureus* or cells was extracted, and complementary DNA (cDNA) was synthesized from total RNA by using the QuantiTect reverse transcription system (Qiagen). Amplification of the resulting cDNA sample utilized the QuantiTect SYBR green PCR kit (Qiagen). The 7500 Sequence Detector (Applied Biosystems) was used to perform reactions in MicroAmp Optical 96-well reaction plates. Oligonucleotides used in this study are presented in Supplementary data-1.

RNA Sequencing

Total RNA was extracted from lung tissues of mice (Qiagen). Nanodrop2000 was used to detect the concentration and purity of the extracted RNA, agarose gel electrophoresis was used to detect RNA integrity, and the Agilent 2100 was used to determine the RIN value. The library was constructed using the Illumina Truseq™ RNA sample prep Kit method, and finally sequenced on the Illumina Novaseq 6000 platform (Shanghai Majorbio Bio-pharm Technology Co., Ltd).

Western blot

Overnight cultures (12-15 hours) were diluted 1:100 into fresh TSB and incubated at 37°C with shaking until stationary phase ($OD_{600nm}=5.0$). 100 μ l of culture filtrate was added into mouse BMDM cultured in a 6-well plate and incubate for 3 hours. The purified SAK(0.5 μ g/ml) protein and lipopolysaccharide (LPS, 0.2 μ g/ml) / ATP (5mM) were incubated with BMDM for 8 hours. Cells were collected after being washed by sterile PBS, and boiled in loading buffer. Then the samples were subjected to western blot experiment with caspase-1 (Abcam) and interleukin-1 β (IL-1 β , Sangon Biotech) antibodies.

Cytokine detection

The level of mouse plasma cytokine was detected by Bio-Plex Pro Mouse Cytokine 23-plex Assay in accordance with the manufacturer's instructions (Bio-Rad, Luminex 200™ System). IL-1 β in the supernatant of mouse BMDM was measured by using ELISA kit (R&D systems). IL-1 β and tumor necrosis factor α (TNF- α) released by THP1 cells were detected by Human IL-1 β or TNF- α ELISA Kit (Sangon Biotech), and interleukin-17A (IL-17A) released by THP1 was measured by Human IL-17 Quantikine HS ELISA Kit (R&D systems).

Reactive Oxygen Species (ROS) Assay

SAK (0.5 μ g/ml) was added into the medium of THP1 cells, and NAC (10 μ M, Sigma) or PBS was added at the same time in 12-well plates of cultured cells at 37°C, and incubated for 5 hours, after which the fluorescent dye 2,7-Dichlorodi-hydrofluorescein diacetate (DCFH-DA, Sigma) was added to the cell culture medium and incubated for

30 min at 37°C in the dark. After washing off the excess dye, the fluorescence signal was collected at an excitation wavelength of 480 nm and emission wavelength of 525 nm, and the cells placed under a fluorescence microscope for observation.

Statistical Analysis

Statistical analyses were performed with Graph-Pad Prism, version 8.0. The comparison of survival curves was carried out by log-rank (Mantel-Cox) test. Unpaired t-tests and two-way ANOVA were used to compare the differences between the variables. All error bars in the graphs show standard deviation ($\pm$ SD). $P < 0.05$ was regarded as statistically significant.

Ethics Statement

All animal experiments were performed in accordance with the laboratory animal care and use guidelines of the Chinese Association for Laboratory Animal Sciences (CALAS). Heparinized venous blood was donated voluntarily by healthy subjects and the use of patient-derived isolates was approved by the ethics committee of Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai (RA-2020-229).

Data availability

RNA-sequence data of this study have been submitted to NCBI (Accession number: SRR16107127, SRR16107128, SRR16107129, SRR16107130). The source data underlying the figures presented in this manuscript are provided in Supplementary Data-2. Extra data are available from the corresponding author upon request.

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501 **Author contributions**

502 Y.W. and N. Z. performed the experiments and wrote the manuscript. Y. J. and Y.L.
 503 analyzed the RNA-sequence data. L.Z. and L.H. performed the statistical analysis. Q.L.
 504 and M.L. designed the experiments and revised the manuscript. All authors reviewed
 505 the manuscript.

506 **Competing interests**

507 The authors declare that they have no conflict of interest.

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Figure 1. The expression level of SAK and its effect on the formation of biofilm. A, The prevalence of *sak* gene in *S. aureus* (SA) epidemic isolates. Deep blue: humans-adapted (HO) isolates; Light blue: livestock-adapted (LA) isolates. Unpaired *t* test was used for statistical analyses between HO-SA and LA-SA. B, qRT-PCR analysis of *sak* gene expression in randomly selected clinical CA-SA (ST398, ST59) and HA-SA (ST239, ST5) isolates, at 4 h of in vitro growth. Relative mRNA levels were calculated using *gyrB* as control and expressed as $2^{(-\Delta\Delta Ct)}$. Unpaired *t* test was used for statistical analyses between CA-SA and HA-SA. C, Measurement of SAK secretion at different incubation times. Two-way ANOVA was used for statistical analyses. D, Biofilm formation ability of CA-SA (ST398, ST59) and HA-SA (ST239, ST5). Unpaired *t* test was used for statistical analyses between CA-SA and HA-SA. E, The effect of adding glucose (GLU), SAK protein, human plasma and mice plasma on the biofilm formation of ST398-WT, *sak* deletion mutant isolate and the complemented isolate. Two-way ANOVA was used for statistical analyses. All data in figure 1 are presented as mean $\pm$ SD and **P*<0.05, ***P*<0.01, ****P*<0.001.

Figure 2. The mouse model of pneumonia shows the role of SAK in ST398 infection. A-G, 2×10^8 CFU of *S. aureus* was pipetted into the nares of anesthetized C57BL/6 wild-type mice (A-D) and *CRAMP*^{-/-} mice (E-G) (n=7). A, E, Photographs of lungs and corresponding H&E stained sections of mice 48 hours after infection. B, Immunohistochemical staining (F4/80) was performed on the lung tissue of infected mice to observe the infiltration of macrophages. C, F, Lung wet weight of infected mice. D, G, The left lung was homogenized and plated on TSB agar for CFU determination. Unpaired *t* test was used for statistical analyses between ST398-WT and *sak* deletion mutant isolate infected mice. Data are presented as mean $\pm$ SD and **P*<0.05, ** *P*<0.01.

Figure 3. The activation of innate immune inflammatory response in lung infection of mice. A-G, Total RNA was extracted from lung tissues of mice infected by ST398-WT and *sak* deletion mutants. A, GSEA analysis identified cytokine related genes (IFN- α , IFN- γ , IL-6, IL-1), complement related genes and NLRP3 inflammasome pathway enrichment in the wild type infected mice. B, The level of mouse plasma cytokine was detected by Bio-Plex Pro Mouse Cytokine 23-plex Assay. The data were normalized by the Z score according to the standard deviation from the mean. C, Comparison of the expression levels of NLRP3 inflammasome activation-related molecules between the two groups. The data were normalized by the Z score according to the standard deviation from the mean. D-G, qRT-PCR analysis of *NLRP3*, *caspase-1*, IL-1 β , and IFN- α in the lung tissues of wild-type or *sak* deletion mutants infected mice. Relative mRNA levels were calculated using β -actin as control and expressed as $2^{-(\Delta\Delta Ct)}$. Unpaired *t* test was used for statistical analyses and data are presented as mean $\pm$ SD and **P* < 0.05, ** *P* < 0.01. See Supplementary data-1 for the abbreviated list of cytokines or genes in Figure 3B and Figure 3C.

Figure 4. The promotion of SAK on NLRP3 inflammasome activation and cytokine expression. A-J, BMDM are incubated with bacterial secretion supernatant or SAK protein. A-F, The mRNA of the cells was extracted and gene expression relative to the negative control group was detected by qRT-PCR after reverse transcription. Relative mRNA levels were calculated using β -actin as internal control and expressed as $2^{-(\Delta\Delta Ct)}$. Unpaired t-test was used to compare the differences between the variables. G: Immunoblot analysis of the cell lysates from BMDM of wild-type mice. H: Secreted IL-1 β from BMDM was detected by ELISA. Unpaired t-test was used to compare the differences between the variables. I: Immunoblot analysis of the cell lysates from BMDM of *caspase1*^{-/-} mice. J: Secreted IL-1 β from BMDM of *caspase1*^{-/-} mice was detected by ELISA. Unpaired t-test was used to compare the differences between the variables. All data are presented as mean $\pm$ SD and * P <0.05, ** P <0.01, *** P <0.001.

Figure 5. Inhibition of the NLRP3 inflammasome can reduce lung infection in mice.
A-D: 10^9 CFU of *S. aureus* was pipetted into the nares of anesthetized C57BL/6 mice (n=6), and 50 mg/kg MCC950 sodium (NLRP3 inflammasome inhibitor) or PBS was injected intraperitoneally to mice two hours before infection. A, C, Survival curves were compared using a log-rank (Mantel-Cox) test. B, D, Tissue sections for HE staining and immunohistochemical staining (IL-1 β). ** $P < 0.01$

Figure 6. SAK exerts a pro-inflammatory effect on THP1 cells, and can promote the release of IL-1 β by increasing the production of ROS.

A-C, Cultured THP1 cells (human monocyte derived cell line) were incubated with bacterial secretion supernatant. The physiologically relevant concentrations of specific cytokine proteins in THP1 cell culture supernatants were detected by ELISA kit. Unpaired t-test was used to compare the differences between the variables. D-E, Detection of the role of ion flux, ROS production and lysosomal damage in the promotion of IL-1 β expression and secretion by SAK. D. The expression level of IL-1 β gene in THP1 cells treated with SAK protein or ST398 culture filtrate relative to the negative control group. Relative mRNA levels were calculated using *GAPDH* as internal control and expressed as $2^{-(\Delta\Delta Ct)}$. Unpaired t-test was used to compare the differences between the variables. E. The secretion of IL-1 β in THP1 cells treated with ST398 culture filtrate was detected by ELISA. Unpaired t-test was used to compare the differences between the variables. F. Reactive Oxygen Species Assay. The fluorescence signal was collected at the excitation wavelength of 488 nm and the emission wavelength of 525 nm. Unpaired t-test was used to compare the differences between the variables. All data are presented as mean $\pm$ SD and * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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608 **Figure 7. The role of SAK in *Staphylococcus aureus* infection.**

- 609 ① SAK can neutralize AMPs and regulate fibrinolysis.
610 ② SAK promotes transcription of inflammation-related genes and activation of
611 NLRP3 inflammasome.

612 Research has shown that SAK can promote the activation of plasminogen in specific
613 hosts (human, rabbits, and sheep) and exert its fibrinolytic function (blue arrows). The
614 epithelium expresses antimicrobial peptides to kill extracellular bacteria. Studies have
615 shown that SAK can bind to α -defensins, human cathelicidin LL-37 and mouse
616 CRAMP to regulate fibrinolysis and evade innate immunity defenses (black arrows). In
617 this study, we demonstrate that SAK plays an important role in acute necrotizing
618 pneumonia by promoting the generation of ROS and activating NLRP3 inflammasomes
619 (red arrows). Furthermore, we speculate through preliminary studies that SAK can
620 promote the transcription of inflammatory response-related molecules and the release
621 of cytokines, help recruit polymorphonuclear leukocytes (PMNs) and contribute to a
622 more active state of inflammatory response in the host. This figure was drawn by using
623 BioRender (<https://app.biorender.com/>).

624

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