Journal of Microbiology, Immunology and Infection 55 (2022) 662—670

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.e-jmii.com

Original Article

Attenuation of virulence in multiple serotypes (M1, M3, and M28) of Group A *Streptococcus* after the loss of secreted esterase

Xiaolan Zhang, Yuan Zhao, Yue Wang, Minghui Cai, Yingli Song, Hui Zhu*

College of Basic Medical Sciences, Harbin Medical University, Harbin, China

Received 20 May 2021; received in revised form 21 July 2021; accepted 15 September 2021
Available online 11 October 2021

KEYWORDS
Group A *Streptococcus* (GAS);
Virulence;
Streptococcal secreted esterase (Sse);
Neutrophil;
Cytokine

Abstract *Introduction:* Group A *Streptococcus* (GAS) can produce streptococcal secreted esterase (Sse), which inhibits neutrophil recruitment to the site of infection and is crucial for GAS pathogenesis. As an effective esterase, Sse hydrolyzes the sn-2 ester bond of human platelet-activating factor, inactivating it and abolishing its ability to recruit neutrophils.
Objectives: The purpose of this study was to investigate the effects of *sse* deletion on the virulence of multiple serotypes of GAS.
Methods: Isogenic strains that lack the *sse* gene (Δ*sse*) were derived from the parent strains MGAS5005 (serotype M1, CovRS mutant), MGAS2221 (serotype M1, wild-type CovRS), MGAS315 (serotype M3, CovRS mutant) and MGAS6180 (serotype M28, wild-type CovRS) and were used to study the differences in virulence and pathogenicity of GAS serotypes.
Results: In a subcutaneous infection model, mice infected with MGAS5005$^{\Delta sse}$ exhibited higher survival rates but decreased dissemination to the organs compared with mice infected with MGAS5005. When mice were infected with the four Δ*sse* mutants, the MPO activity and IFN-γ, TNF-α, IL-2 and IL-6 levels increased, but the skin lesion sizes decreased. In an intraperitoneal infection model, the absence of Sse significantly reduced the virulence of GAS, leading to increased mouse survival rates and decreased GAS burdens in the organs in most of the challenge experiments. In addition, the numbers of the four Δ*sse* mutants were greatly reduced 60 min after incubation with isolated rat neutrophils.
Conclusion: Our results suggest that Sse participates in the pathogenesis of multiple GAS serotypes (MGAS5005, MGAS2221, MGAS315 and MGAS6180), particularly the hypervirulent CovS

* Corresponding author. No.157 Baojian Road, Harbin, 150081, China.
E-mail address: zhuhui@ems.hrbmu.edu.cn (H. Zhu).

https://doi.org/10.1016/j.jmii.2021.09.008
1684-1182/Copyright © 2021, Taiwan Society of Microbiology. Published by Elsevier Taiwan LLC. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

mutant strains MGAS5005 and MGAS315. These strain differences were positively correlated with the virulence of the serotype.
Copyright © 2021, Taiwan Society of Microbiology. Published by Elsevier Taiwan LLC. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Introduction

Group A *Streptococcus* (GAS) is a human specific pathogen that often causes pharyngitis and impetigo. However, rheumatic heart disease and invasive GAS infections that are related to the onset of mild infections lead to over 500,000 deaths every year worldwide.[1—4] Although the antibiotic drug penicillin can cure most GAS infections, these infections are still considered to be life-threatening. The virulence of GAS is closely related to secreted extracellular virulence factors.[5—8] The well-studied virulence factors are the hemolysins SLO and SLS, which can form hydrophilic pores to destroy erythrocytes, leukocytes and other tissues.[9,10] Another important virulence factor, streptokinase, can activate plasminogen, which generates the plasmin that activates metalloproteinases to dissolve fibrin and degrade the extracellular matrix. Streptokinase allows GAS to disseminate from the infection site to distant locations within the host.[11] Certainly, there are a variety of other secreted virulence factors, such as cysteine proteinase SpeB, CAMP factor, DNases, inhibitors of complement, Ig-binding proteins and esterase.[12—15] Streptococcal secreted esterase (Sse) is a carboxylic acid esterase that can hydrolyze human platelet-activating factor (PAF).[16,17] Produced by endothelial cells, neutrophils, macrophages and granular eosinophils, PAF serves as a phospholipid mediator that recruits immunocytes to the infection site.[18,19] Thus, Sse inhibits recruitment of neutrophils to the infection site, which provides a mechanism by which for GAS evades the innate immune system. Sse has been demonstrated to participate in subcutaneous GAS infection and dissemination from the skin.[20—22] Our published results have showed that Sse immunization has an immunoprotective effect against multiple serotypes of Group A Streptococcus.[23] However, the immune response and toxicity with or without Sse are still need to be further studied.

It is known that some virulence factors can vary among GAS serotypes and strains, since some genomes do not contain the genes encoding those virulence factors, or the genes have mutations that do not allow for normal transcription or translation.[12,24—26] Therefore, it is crucial to investigate whether Sse participates in the pathogenesis of GAS and influences the virulence of different serotypes. Sse is relatively conserved among serotypes and has two distinct variants, complexes I and II.[20] Complex I includes Sse proteins secreted by the serotypes M1, M2, M3, M5, M6, M12 and M18, whereas complex II contains those secreted by the serotypes M4, M28, and M49. In previous study, we found that the *sse* knockout led to the reduced virulence in serotypes M1 (MGAS5005 and MGAS2221).[16,17,21] These results encouraged us to speculate whether the *sse* knockout can also attenuate the virulence of other serotypes, including hypervirulent epidemic M3 strain in Europe and America (MGAS315) and hypovirulent M28 strain producing Sse of complex II (MGAS6180). The aim of our study was to investigate whether the *sse* knockout could reduce the virulence of different virulent serotypes of GAS and besides the mechanism of hydrolyzing PAF to impede neutrophil recruitment, whether the *sse* knockout could influence phagocytosis by neutrophils and the amounts of released cytokines. In this study, we compared isogenic strains lacking the *sse* gene (Δ*sse*) with the parent serotypes M1, M3, and M28 to determine the effect of Sse on the virulence of multiple GAS serotypes. Understanding the role of Sse in the disease process can provide valuable information for the development of new therapeutic agents that target Sse.

Methods

Bacterial strains and growth conditions

The hypervirulent M1 CovRS mutant strain MGAS5005, the wild-type M1 strain MGAS2221, the hypervirulent M3 CovRS mutant strain MGAS315, and the wild-type M28 strain MGAS6180 were considered the wild-type strains in this study. These wild-type strains and their Δ*sse* mutants were propagated in Todd-Hewitt broth (BD Bioscience) supplemented with 0.2% (w/v) yeast extract (Amresco) at 37 °C and 5% CO_2. The bacteria were grown to logarithmic phase in THY ($OD_{600} \approx 0.8$), pelleted, washed and resuspended in phosphate-buffered saline (PBS). When recording the growth curves, all these strains were cultured from the same initial quantity ($OD_{600} = 0.05$).

Construction of the Δ*sse* strains

Strains carrying an in-frame allelic replacement of *sse* were generated from the wild-type strains as previously described.[17] The knock-out fragments are shown in the Table 1. The Δ*sse* strains were verified by polymerase chain

Table 1 The knock-out fragments in four strains.

	The full length of *sse*	The length of the knock-out fragment
$MGAS5005^{\Delta sse}$	1—987 bps	187—687 bps
$MGAS2221^{\Delta sse}$	1—987 bps	169—784 bps
$MGAS315^{\Delta sse}$	1—987 bps	169—787 bps
$MGAS6180^{\Delta sse}$	1—1002 bps	188—784 bps

reactions and sequencing (Life Technologies, Shanghai). The deletion of *sse* did not disrupt the open reading frame.

Mouse infection experiments

CD1 mice (18—22 g) were purchased from the Department of Experimental Animals of Harbin Medical University. In the subcutaneous infection model (SC), the mice were subcutaneously inoculated with 0.2 ml ($\approx 2.0 \times 10^8$ CFU) of a bacterial suspension in PBS. The mice (10 mice/group, eight total groups) were monitored daily for 10 days to determine the survival rates. The remaining mice in each group were euthanized at 24 h post inoculation to measure skin lesion sizes (6 mice/group), GAS burdens in the spleen, liver, and kidney (6 mice/group), myeloperoxidase (MPO) activity (5 mice/group) and cytokine levels (4 mice/group) at the infection sites. In the intraperitoneal infection model (IP), the mice were intraperitoneally injected with 0.2 ml ($\approx 2.0 \times 10^8$ CFU) of bacterial suspension for each GAS strain. The mice (10 mice/group, eight groups in total) were monitored to determine the survival rates. The other mice (6 mice/group) in each group were euthanized at 24 h after inoculation, or within 24 h if they were moribund, to collect the spleen, liver, and kidney to measure the GAS burdens. All the protocols were approved by the Institutional Research Board of Harbin Medical University.

Detection of myeloperoxidase (MPO) activity

The entire skin lesions were excised from the mice infected with all the Δ*sse* mutants and their wild-type strains and homogenized. The level of MPO activity (U/total) was determined using an MPO assay kit (Nanjing Jiancheng Bioengineering Institute) with the aid of a microplate reader (SpectraMax).

Phagocytosis assay

Rat neutrophils were isolated from 3 ml of circulating blood by using an isolation solution of mononuclear cells (HISTOPAQUE-1083, Sigma) for the in vitro measurements of GAS resistance to neutrophil-mediated killing. Overnight cultures of GAS (eight strains in total, n = 6 cultures/strain) were diluted and mixed with 2×10^4 neutrophils in 1 ml PBS [multiplicity of infection (MOI) = 10:1]. The samples were incubated at 37 °C for 3 h. The mixed samples were spread onto THY plates, and the numbers of bacteria were calculated at 0, 30, 60, 120, 180 min after incubation.

Cytokine measurement by ELISA

Twenty-four hours post infection, the skin lesions of the mice were excised and homogenized. The homogenates were centrifuged to obtain the supernatant. The levels of TNF-α, IL-2, IL-6, and IFN-γ in the supernatants were quantified using enzyme-linked immunosorbent assay (ELISA) kits (Elabscience Biotechnology Co., Ltd.).

Statistical analysis

The log-rank test was used to analyze the survival rate of the mice. The data were analyzed by Student's t-test or two-way ANOVA with Fisher's LSD multiple comparisons test. All the figures were generated using GraphPad Prism version 6.0. A P value < 0.05 was considered statistically significant.

Results

The deletion of *sse* does not impair growth in culture medium

First, it was necessary to compare the growth of the four Δ*sse* mutants with the growth of their wild-type strains in THY medium. All the Δ*sse* mutants could proliferate normally in vitro. There were obvious lag phases, logarithmic phases, and stationary phases in each bacterial growth curve (Fig. 1). Thus, the Δ*sse* mutants exhibit no growth defects in vitro.

The deletion of *sse* significantly reduces the virulence of MGAS5005 in the subcutaneous infection model

To examine whether the deletion of *sse* can attenuate the virulence of GAS and whether such attenuation can vary among strains, mice were infected with four Δ*sse* mutant strains and their parent strains M1, M3, and M28. Ten days after subcutaneous challenge, the survival rate of the mice infected with MGAS5005$^{\Delta sse}$ was 80.0%, whereas the survival rate of the mice infected with MGAS5005 was 0% ($P < 0.0001$, Fig. 2A). Similarly, the mice infected with MGAS315$^{\Delta sse}$ had a higher survival rate than the mice infected with MGAS315 ($P = 0.0204$, Fig. 2C). However, only one mouse died when infected with MGAS2221 or MGAS2221$^{\Delta sse}$ and all the mice were still alive after infection with MGAS6180 or MGAS6180$^{\Delta sse}$ after 10 days (Fig. 2B, D). The difference in the survival rate was not significant for these two groups. These results indicate that the deletion of *sse* probably affects the virulence of different GAS serotypes in the subcutaneous infection model to varing degrees.

The deletion of *sse* enhances the recruitment of neutrophils and reduces skin lesions

It was necessary to determine the number of neutrophils recruited after infection based on MPO activity. As shown in Fig. 3A, the MPO activity of the mice infected with the four Δ*sse* mutants was higher than that of the mice infected with the wild-type strains, and the differences were significant except for that between the mice infected with MGAS315 or MGAS315$^{\Delta sse}$. The number of neutrophils is proportional to the MPO activity. This result indicated that once *sse* was deleted, more neutrophils were recruited to

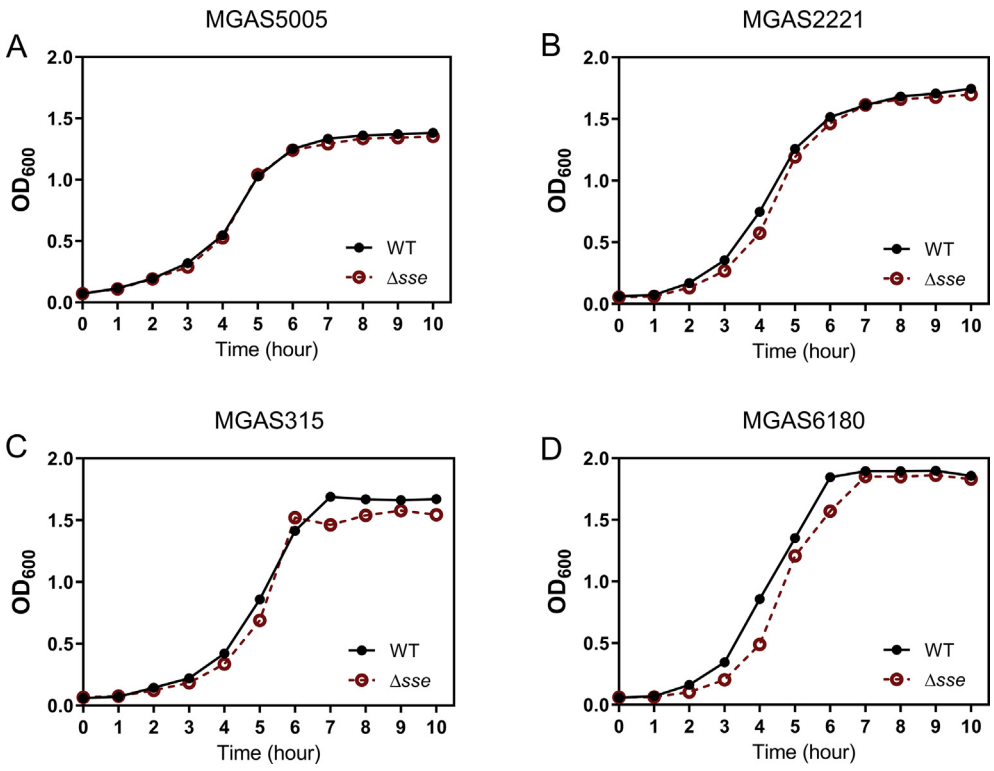

Figure 1. Bacterial growth curves of all Δ*sse* mutants and their wild type strains. (A) MGAS5005 and MGAS5005$^{\Delta sse}$; (B) MGAS2221 and MGAS2221$^{\Delta sse}$; (C) MGAS315 and MGAS315$^{\Delta sse}$; (D) MGAS6180 and MGAS6180$^{\Delta sse}$.

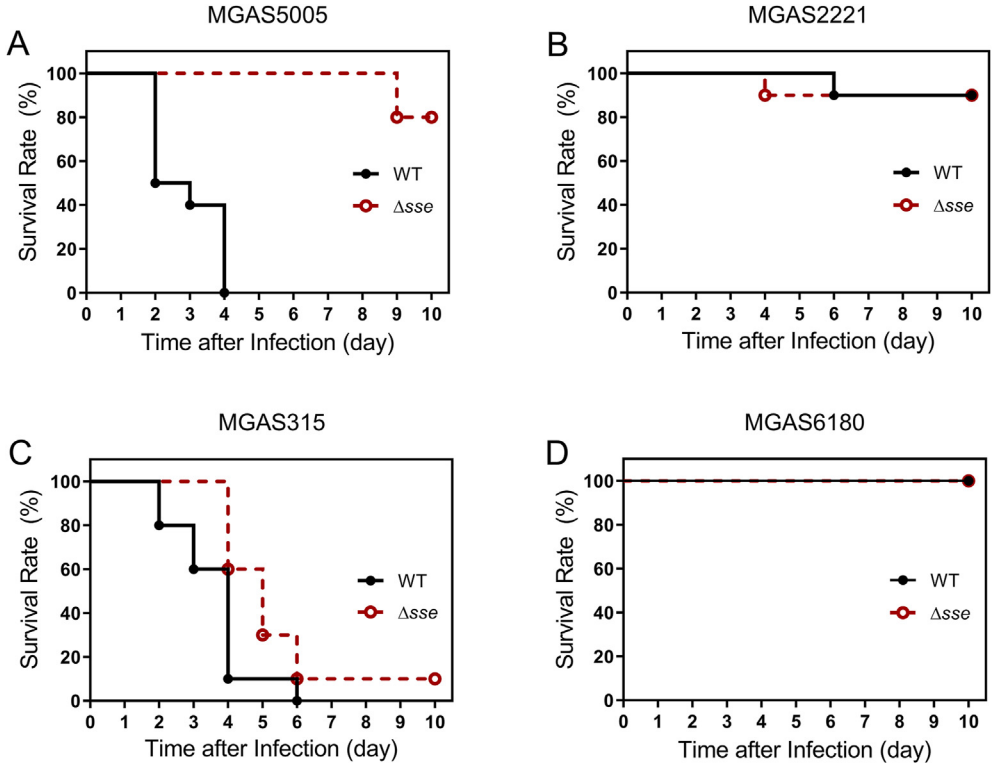

Figure 2. The survival rate curves of mice subcutaneously infected with group A *Streptococcus*. CD1 mice were inoculated in the back with 2.0 × 10^8 CFU of the bacterial suspension. Then, the mice were observed over 10 days. Statistical significance was determined by the log-rank test. (A) Mice infected with MGAS5005 and MGAS5005$^{\Delta sse}$; (B) Mice infected with MGAS2221 and MGAS2221$^{\Delta sse}$; (C) Mice infected with MGAS315 and MGAS315$^{\Delta sse}$; (D) Mice infected with MGAS6180 and MGAS6180$^{\Delta sse}$.

the infection sites. Furthermore, we measured the area of the skin lesions to compare the level of skin invasion. The mean lesion sizes of the mice infected with the four Δ*sse* mutants were smaller than those of the mice infected with the wild-type strains, and the differences were significant except for that between the mice infected with MGAS6180 and MGAS6180$^{\Delta sse}$ (Fig. 3B). The reduced lesion size was proportional to the reduced level of skin invasion, which was limited by the increased recruitment of neutrophils. These data suggest that the deletion of *sse* enhances the recruitment of neutrophils and reduces the size of skin lesions in mice infected with multiple GAS serotypes.

Furthermore, the numbers of bacteria in the organs were calculated to compare the dissemination of the different GAS serotypes. As shown in Fig. 4, there was a significant reduction in the bacterial loads in the mice subcutaneously infected with MGAS5005$^{\Delta sse}$. However, there was no obvious difference when the mice were infected with hypovirulent MGAS2221$^{\Delta sse}$ or MGAS6180$^{\Delta sse}$, which was in accordance with the rates of survival after subcutaneous challenge.

The deletion of *sse* significantly reduces the virulence of multiple serotypes in the intraperitoneal infection model

We used another infection route to observe the virulence of GAS in vivo. After peritoneal inoculation with MGAS5005, the majority of the mice died within the first day, whereas the survival rate of the mice infected with the same amount of MGAS5005$^{\Delta sse}$ was as high as 90% ($P = 0.0003$, Fig. 5A). All the mice infected with MGAS315 died within the first day, but the survival rate of the mice infected with MGAS315$^{\Delta sse}$ was 60% ($P = 0.0236$, Fig. 5C). Furthermore, the survival rate of the mice infected with MGAS2221$^{\Delta sse}$ was sixfold higher than that of the mice infected with MGAS2221 ($P = 0.0165$, Fig. 5B), and the survival rate of the mice infected with MGAS6180$^{\Delta sse}$ was fourfold higher than that of the mice infected with MGAS6180 ($P = 0.0039$, Fig. 5D). The absence of Sse greatly attenuates the virulence of multiple GAS serotypes in the intraperitoneal infection model. Moreover, there were obvious decreases in bacterial CFUs in the mice infected with the Δ*sse* mutants compared with the mice infected with the wild-type strains (Fig. 6). These results demonstrate that the deletion of *sse* reduces the systemic dissemination of GAS to other organs, which contributes to the enhanced the survival rate of mice.

The deletion of *sse* reduces the ability of GAS to resist phagocytosis

Further investigation was performed to compare the neutrophil-mediated killing of the different GAS strains. Neutrophils were extracted from fresh rat blood and coincubated with four Δ*sse* mutants and their wild-type strains. The numbers of all the strains decreased after coincubation with neutrophils over time, but this was particularly obvious for the Δ*sse* mutants (Fig. 7). After 60 min of incubation, a significant difference between numbers of the Δ*sse* mutants and the wild-type strains was observed. The survival percentage of MGAS5005 was 64.4%, which was higher than the 50.6% survival percentage of MGAS5005$^{\Delta sse}$ at 60 min. Similarly, the survival percentage of MGAS315 was 75.6%, which was higher than the 58.7% survival percentage of MGAS5005$^{\Delta sse}$ 60 min post incubation. For MGAS2221 and MGAS6180, the survival percentages at 60 min decreased greatly to 42.0% and 32.3%, but were still higher than the 20.7% and 15.5% survival percentages of MGAS2221$^{\Delta sse}$ and MGAS6180$^{\Delta sse}$. Therefore, the Δ*sse* mutants were less able to resist being killed by neutrophils. These results illustrate that Sse contributes to resisting phagocytosis by neutrophils at the early stage of GAS infection.

The amounts of released cytokines increased in MGAS5005$^{\Delta sse}$ -infected skin

To eliminate pathogens, immunocytes use different strategies, including engulfing invaders or releasing enzymes and cytokines, at inflammatory sites. These cytokines play numerous crucial functions. After 24 h of infection, the levels of IFN-γ, TNF-α, IL-2, and IL-6 at the skin infection sites were much higher in the mice infected with the Δ*sse* mutants (Fig. 8). There was an obvious difference between the mice infected with M1 type MGAS5005 and MGAS5005$^{\Delta sse}$, but there was no significant difference in the production of IFN-γ or IL-2 between the mice infected with MGAS2221 and MGAS2221$^{\Delta sse}$, another M1 type of GAS. This demonstrates that different virulent serotypes elicit immune responses to varying degrees and in different patterns, and these increased cytokines help to establish

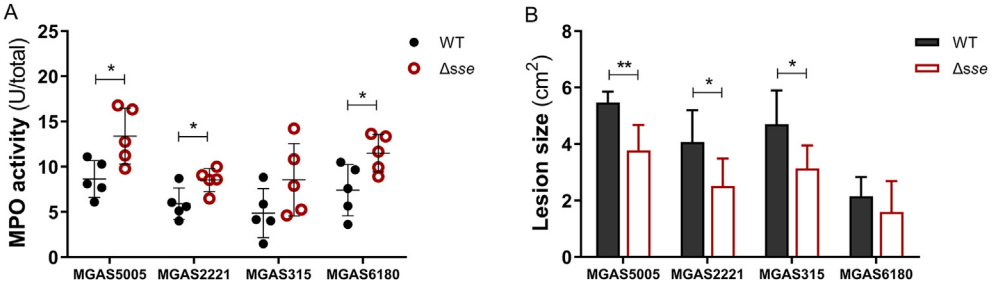

Figure 3. Skin invasion caused by multiple GAS serotypes at 24 h post inoculation. (A) MPO activity in the skin infection site, n = 5. (B) Skin lesion size of mice infected with MGAS5005, MGAS2221, MGAS315 and MGAS6180, n = 6. All the data are represented as the mean ± SD. These data were represented as the mean ± SD, n = 6. *$P < 0.05$, **$P < 0.01$.

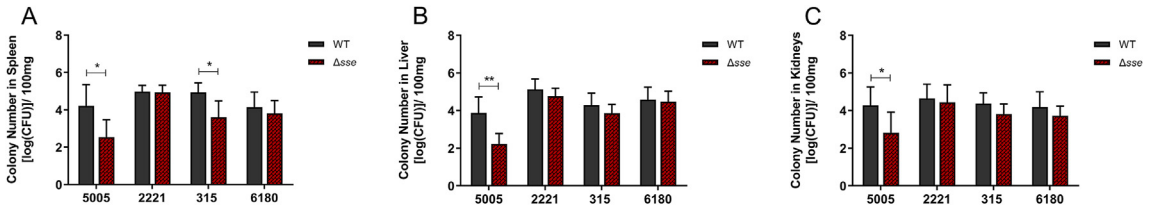

Figure 4. Bacterial CFU numbers in the spleen, liver and kidneys 24 h after subcutaneous challenge. The organ homogenates were serially diluted in PBS and plated on THY agar plates for the enumeration of bacterial CFUs (n = 6) from (A) spleen, (B) liver, and (C) kidneys. All the data are represented as the mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$.

immunological recognition and resist GAS infection, which probably more effectively eliminates the Δ*sse* mutants at the local site of infections.

Discussion

Sse has been demonstrated in previous studies to participate in subcutaneous infection and in dissemination from the skin to other organs.[16,17,20–22] However, it is unknown whether the attenuation of virulence in the absence of Sse can vary from strain to strain. There are two variant Sse complexes: complex I and complex II. Complex I includes the serotypes M1, M2, M3, M5, M6, M12, and M18, whereas complex II includes the serotypes M4, M28, and M49. In this study, we compared isogenic strains lacking the *sse* gene (Δ*sse*) with their parent strains M1, M3, and M28 to determine the difference in the attenuation of virulence.

First, it was necessary to compare the growth of the strains in the medium to exclude the effects of growth defects after the deletion of *sse*. All four Δ*sse* mutants can grow normally in vitro. Then, we observed the 10-day survival of mice infected with different GAS strains and found that compared with the mice infected with the wild-type strains, the mice infected with the Δ*sse* mutants (MGAS5005$^{\Delta sse}$ and MGAS315$^{\Delta sse}$) had higher survival rates, but there was no significant change in the survival rates of the mice infected with the hypovirulent serotypes (MGAS2221$^{\Delta sse}$ or MGAS6180$^{\Delta sse}$). Furthermore, we calculated the bacterial loads to compare dissemination in the organs. The mice subcutaneously infected with hypervirulent MGAS5005$^{\Delta sse}$ and MGAS315$^{\Delta sse}$ had fewer bacteria, but similar results were not observed in the mice infected with MGAS2221$^{\Delta sse}$ or MGAS6180$^{\Delta sse}$. These results were in accordance with the survival rates of the subcutaneous infection model. Next, we used another challenge route,

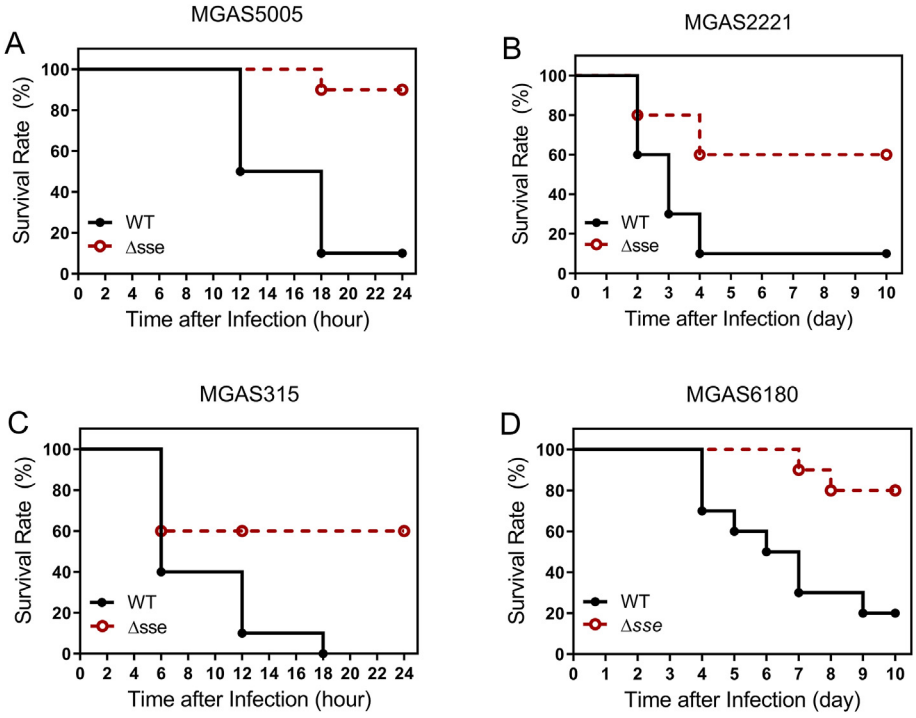

Figure 5. The survival rate curves of mice intraperitoneally infected with group A *Streptococcus*. CD1 mice were injected with 2.0×10^8 CFU of the bacterial suspension. The survival of mice was observed within 24 h or 10 days. Statistical significance was determined by the log-rank test. (A) Mice infected with MGAS5005 and MGAS5005$^{\Delta sse}$; (B) Mice infected with MGAS2221 and MGAS2221$^{\Delta sse}$; (C) Mice infected with MGAS315 and MGAS315$^{\Delta sse}$; (D) Mice infected with MGAS6180 and MGAS6180$^{\Delta sse}$.

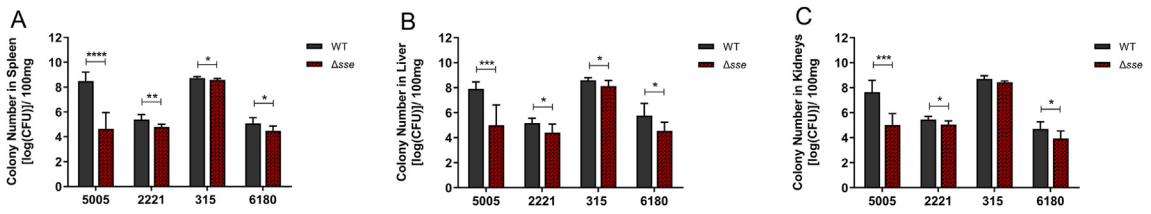

Figure 6. Bacterial CFUs in the spleen, liver and kidneys 24 h after intraperitoneal challenge. The organ homogenates were serially diluted in PBS and plated on THY agar plates for the enumeration of bacterial CFUs (n = 6) in (A) spleen, (B) liver, and (C) kidneys. All the data are represented as the mean ± SD, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

intraperitoneal injection, to observe virulence. In this model, the mortality of the mice was much higher than that of the mice infected subcutaneously on the skin. The intraperitoneally infected mice died more quickly, and there were significant differences between the mice infected with the four Δ*sse* mutants and the wild-type strains. These results demonstrate that Sse participates in the pathogenesis of multiple GAS serotypes to varying degrees.

Previous studies had showed the strong hydrolysis capacity of Sse^{M1} but relatively weak Sse^{M28}.[17] Sse hydrolyzes PAF to impede neutrophil recruitment by the host and promotes the innate immune evasion of GAS.[16–19] Thus, we detected the release of MPO in the skin lesions, which is proportional to the number of recruited neutrophils.[27,28] The MPO activity of the mice infected with the Δ*sse* mutants was higher than that of the mice infected with the wild-type strains. This result indicated that more neutrophils were recruited to the infection sites when mice were infected with Δ*sse* mutants. The reduced virulence was further confirmed by the size of the skin lesions. The average skin lesion sizes of mice infected with the Δ*sse* mutants were smaller than those of mice infected with the wild-type strains. These results illustrated that the deletion of *sse* mitigated skin inflammation and impeded the dissemination of GAS in the skin. There were also differences in subcutaneous GAS infection and dissemination from the skin among serotypes M1, M3 and M28, which indicated the varying activity of Sse in hydrolyzing PAF to inhibit neutrophil recruitment.

Further experiments were conducted to compare the neutrophil-mediated killing of GAS. After 60 min of coincubation with neutrophils, significant differences in the numbers of the Δ*sse* mutants and the wild-type strains were observed, particularly for the hypovirulent serotypes MGAS2221 or MGAS6180. The Δ*sse* mutants were less able to

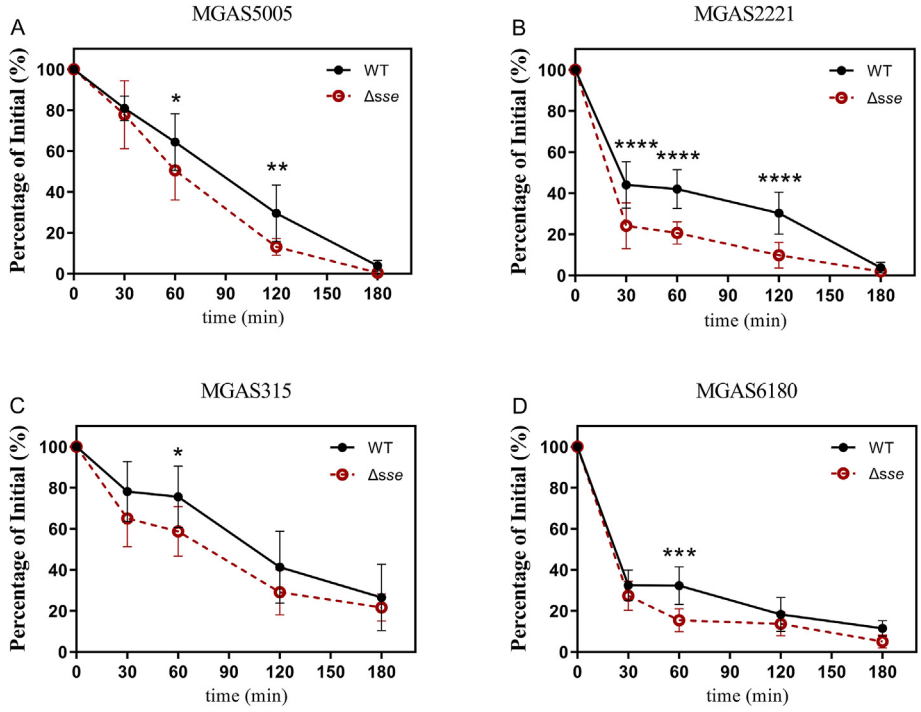

Figure 7. The level of antiphagocytosis activity when incubated with rat neutrophils in vitro. Rat neutrophils were isolated from fresh blood and then coincubated with GAS strains (MOI = 10:1) (n = 6). (A) MGAS5005 and $MGAS5005^{\Delta sse}$. (B) MGAS2221 and $MGAS2221^{\Delta sse}$. (C) MGAS315 and $MGAS315^{\Delta sse}$. (D) MGAS6180 and $MGAS6180^{\Delta sse}$. These data are represented as the mean ± SD, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

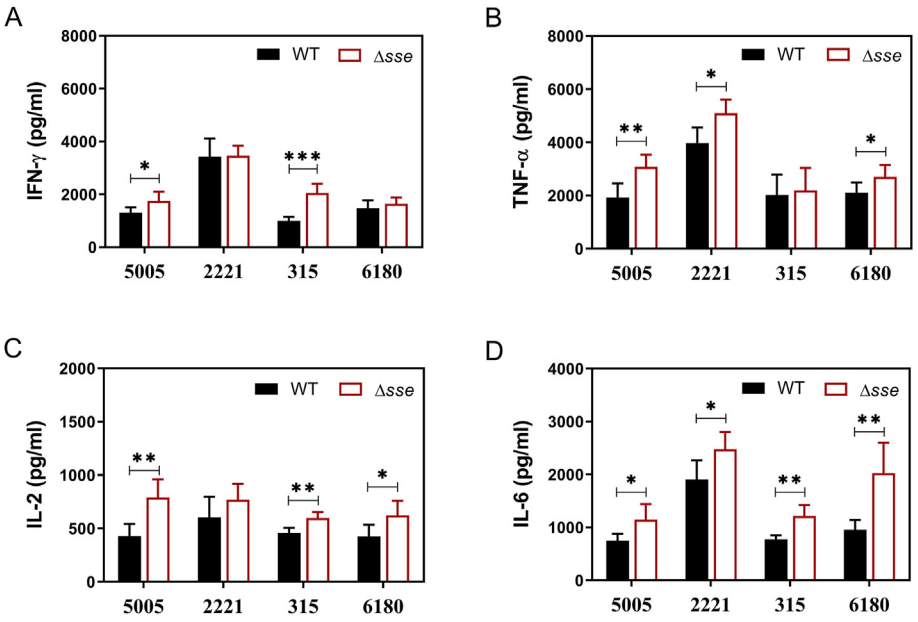

Figure 8. The amounts of cytokines released in the skin infection sites. (A) IFN-γ, (B) TNF-α, (C) IL-2, (D) IL-6. All the data are represented as the mean ± SD (n = 4), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

resist being killed by neutrophils, which showed that Sse contributed to resisting phagocytosis by neutrophils in the first hours of infection. It had differences in resisting the neutrophil-mediated killing among serotypes, which is also an important mechanism in the pathogenesis of GAS. MGAS315 had stronger anti-phagocytosis ability when coincubation with isolated neutrophils than MGAS5005, and MGAS6180 showed the weakest anti-phagocytosis ability. The absence of Sse attenuates the virulence and pathogenicity of different serotypes of GAS to varying degrees, which is also impacted by the infection route. We also examined the immune response mediated by immunocytes other than neutrophils at skin infection sites. These cytokines work together to mediate innate and adaptive immune responses to eliminate GAS in the skin. The amount of cytokines at the skin infection sites was much greater in the mice infected with the Δ*sse* mutants. Both IFN-γ and TNF-α, which are mainly released by macrophages, participate in immune defense and inhibit bacterial proliferation. We found that the amounts of these two cytokines were much more than IL-2 and IL-6, which reflected the activation of macrophages. MGAS2221 elicited more IFN-γ and TNF-α than MGAS315 or MGAS6180. IL-2 plays a key role in regulating T cells and enhances cell-mediated immunity. But no obvious difference of IL-2 was found in M1, M3 and M28, which probably indicated less and unimportant cell-mediated immunity in GAS infection. IL-6 is an interleukin that has both proinflammatory and anti-inflammatory effects. The amounts of IL-6 in hypovirulent MGAS2221 and MGAS6180 were much more than those of hypervirulent MGAS5005 and MGAS315. These results demonstrate that different virulent serotypes elicit immune responses in different patterns, and it is necessary to add more serotypes for accurate conclusions. This study indicates that the *sse* knockout could reduce the virulence of M1, M3 and M28 of GAS and besides the mechanism of hydrolyzing PAF to impede neutrophil recruitment, the *sse* knockout could influence phagocytosis by neutrophils and the immune response to varying degrees among serotypes. As virulence factors do not work in isolation, it will be valuable to determine additional roles of Sse together with other virulence factors in the disease process.

Declaration of competing interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

This work was supported by the grants from National Natural Science Foundation of China (81571957), Heilongjiang Postdoctoral Science Foundation (LBH-Z17154; LBH-Z19175), Heilongjiang Health and Family Planning Commission Research Fund (2018-473).

References

1. Walker Mark J, Barnett Timothy C, McArthur Jason D, Cole Jason N, Gillen Christine M, Henningham Anna, et al. Disease manifestations and pathogenic mechanisms of Group A Streptococcus. *Clin Microbiol Rev* 2014;**27**(2):264—301.
2. Lynskey NN, Jauneikaite E, Li HK, Zhi X, Turner CE, Mosavie M, et al. Emergence of dominant toxigenic M1T1 Streptococcus pyogenes clone during increased scarlet fever activity in England: a population-based molecular epidemiological study. *Lancet Infect Dis* 2019;**S1473—3099**(19):30446—53.

3. Hua Chun-Zhen, Yu Hui, Xu Hong-Mei, Yang Lin-Hai, Lin Ai-Wei, Lyu Qin, et al. A multi-center clinical investigation on invasive Streptococcus pyogenes infection in China, 2010—2017. *BMC Pediatr* 2019;**19**(181):1—6.
4. Dickson C, Pham MT, Nguyen V, Brubacher C, Silverman MS, Khaled K, et al. Community outbreak of invasive Group A Streptococcus infection in Ontario, Canada. *Can Comm Dis Rep* 2018;**44**(7—8):182—8.
5. Sarkar P, Sumby P. Regulatory gene mutation: a driving force behind Group A Streptococcus strain- and serotype-specific variation. *Mol Microbiol* 2017;**103**(4):576—89.
6. Hamada S, Kawabata S, Nakagawa I. Molecular and genomic characterization of pathogenic traits of Group A Streptococcus pyogenes. *Proc Jpn Acad Ser B Phys Biol Sci* 2015;**91**(10): 539—59.
7. Laabei Maisem, Ermert David. Catch me if you can: Streptococcus pyogenes complement evasion strategies. *J Innate Immun* 2018;**11**(1):3—12.
8. Remmington A, Turner CE. The DNases of pathogenic Lancefield Streptococci. *Microbiology* 2018;**164**(3):242—50.
9. Uchiyama S, Döhrmann S, Timmer AM, Dixit N, Ghochani M, Bhandari T, et al. Streptolysin O rapidly impairs neutrophil oxidative burst and antibacterial responses to Group A Streptococcus. *Front Immunol* 2015;**16**(6):1—22.
10. Flaherty RA, Donahue DL, Carothers KE, Ross JN, Ploplis VA, Castellino FJ, et al. Neutralization of streptolysin S-dependent and independent inflammatory cytokine IL-1β activity reduces pathology during early Group A Streptococcal skin infection. *Front Cell Infect Microbiol* 2018;**8**:211.
11. Glinton K, Beck J, Liang Z, Qiu C, Lee SW, Ploplis VA, et al. Variable region in streptococcal M-proteins provides sTable binding with host fibrinogen for plasminogen-mediated bacterial invasion. *J Biol Chem* 2017;**292**(16):6775—85.
12. Hynes Wayne, Sloan Melanie. *Secreted extracellular virulence factors. Streptococcus pyogenes: basic biology to clinical manifestations*. Oklahoma: The University of Oklahoma Health Sciences Center Library; 2016. p. 1—23.
13. Terao Yutaka. The virulence factors and pathogenic mechanisms of Streptococcus pyogenes. *J Oral Biosci* 2012;**54**: 96—100.
14. Westman J, Chakrakodi B, Snäll J, Mörgelin M, Bruun Madsen M, Hyldegaard O, et al. Protein SIC secreted from Streptococcus pyogenes forms complexes with extracellular histones that boost cytokine production. *Front Immunol* 2018;**9**:236.
15. Sumitomo T, Mori Y, Nakamura Y, Honda-Ogawa M, Nakagawa S, Yamaguchi M, et al. Streptococcal cysteine protease-mediated cleavage of desmogleins is involved in the pathogenesis of cutaneous infection. *Front Cell Infect Microbiol* 2018;**8**(10):1—10.
16. Liu Mengyao, Zhu Hui, Li Jinquan, Garcia Cristiana C, Feng Wenchao, Kirpotina Liliya N, et al. Group A Streptococcus secreted esterase hydrolyzes platelet-activating factor to impede neutrophil recruitment and facilitate innate immune evasion. *PLoS Pathog* 2012;**8**(4):e1002624.
17. Liu Guanghui, Liu Mengyao, Xie Gang, Lei Benfang. Characterization of streptococcal platelet-activating factor acetylhydrolase variants that are involved in innate immune evasion. *Infect Immun* 2013;**81**(9):3128—38.
18. Chaithra Vyala Hanumanthareddy, Jacob Shancy Petsel, Lakshmikanth Chikkamenahalli Lakshminarayana, Sumanth Mosale Seetharam, Abhilasha Kandahalli Venkataranganayaka, Chen Chu-Huang, et al. Modulation of inflammatory platelet-activating factor (PAF) receptor by the acyl analogue of PAF. *J Lipid Res* 2018;**59**(11):2063—74.
19. Liu Gang, Mateer Sean W, Hsu Alan, Goggins Bridie J, Tay Hock, Mathe Andrea, et al. *Platelet activating factor receptor regulates colitis-induced pulmonary inflammation through the NLRP3 inflammasome*. 2019. https://doi.org/10.1038/s41385-019-0163-3.
20. Liu Mengyao, Zhu Hui, Zhang Jinlian, Lei Benfang. Active and passive immunizations with the streptococcal esterase Sse protect mice against subcutaneous infection with Group A Streptococci. *Infect Immun* 2007;**75**(7):3651—7.
21. Zhu Hui, Liu Mengyao, Sumby Paul, Lei Benfang. The secreted esterase of Group A Streptococcus is important for invasive skin infection and dissemination in mice. *Infect Immun* 2009; **77**(12):5225—32.
22. Liu Mengyao, Feng Wenchao, Zhu Hui, Lei Benfang. A neutralizing monoclonal IgG1 antibody of platelet-activating factor acetylhydrolase SsE protects mice against lethal subcutaneous Group A Streptococcus infection. *Infect Immun* 2015;**83**(7):2796—805.
23. Zhang Xiaolan, Wei Deqin, Zhao Yuan, Zhong Zhaohua, Wang Yue, Song Yingli, et al. Immunization with a secreted esterase protects mice against multiple serotypes (M1, M3, and M28) of Group A *Streptococcus*. *Front Microbiol* 2020;**11**:565.
24. Happonen Lotta, Hauri Simon, Birkedal Gabriel Svensson, Karlsson Christofer, de Neergaard Therese, Khakzad Hamed, et al. A quantitative Streptococcus pyogenes—human protein—protein interaction map reveals localization of opsonizing antibodies. *Nat Commun* 2019;**10**:2727.
25. Hirose Yujiro, Yamaguchi Masaya, Okuzaki Daisuke, Motooka Daisuke, Hamamoto Hiroshi, Hanada Tomoki, et al. Streptococcus pyogenes transcriptome changes in inflammatory environment of necrotizing fasciitis. *Appl Environ Microbiol* 2019. https://doi.org/10.1128/AEM.01428-19.
26. Nye Taylor M, Jacob Kristin M, Holley Elena K, Nevarez Juan M, Dawid Suzanne, Simmons Lyle A, et al. DNA methylation from a type I restriction modification system influences gene expression and virulence in Streptococcus pyogenes. *PLoS Pathog* 2019;**15**(6):e1007841.
27. Carr AC, Spencer E, Hoskin TS, Rosengrave P, Kettle AJ, Shaw G. Circulating myeloperoxidase is elevated in septic shock and is associated with systemic organ failure and mortality in critically ill patients. *Free Radic Biol Med* 2019; **S0891—5849**(19):31559-X.
28. Xiang Y, Jin C, Wang W, Wang Z, Huang Y, Fan F, et al. The critical role of myeloperoxidase in Streptococcus pneumoniae clearance and tissue damage during mouse acute otitis media. *Innate Immun* 2017;**23**(3):296—306.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2021.09.008.