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Streptolysin S induces proinflammatory cytokine expression in calcium ion-influx-dependent manner

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ABSTRACT

Anginosus group streptococci (AGS) are opportunistic pathogens that reside in the human oral cavity. The β-hemolytic strains of *Streptococcus anginosus* subsp. *anginosus* (SAA) produce streptolysin S (SLS), a streptococcal peptide hemolysin. In recent clinical scenarios, AGS, including this species, have frequently been isolated from infections and disorders beyond those in the oral cavity. Consequently, investigating this situation will reveal the potential pathogenicity of AGS to ectopic infections in humans. However, the precise mechanism underlying the cellular response induced by secreted SLS and its relevance to the pathogenicity of AGS strains remain largely unknown. This study aims to elucidate the mechanism underlying the host cellular response of the human acute monocytic leukemia cell line THP-1 to secreted SLS. In THP-1 cells incubated with the culture supernatant of β-hemolytic SAA containing SLS as the sole cytotoxic factor, increased Ca^{2+} influx and elevated expression of proinflammatory cytokines were observed. Significantly reduced expression of SLS-dependent upregulated cytokine genes under Ca^{2+}-chelating conditions suggests that Ca^{2+} influx triggers SLS-dependent cellular responses. Furthermore, SLS-dependent enhanced expression of IL-8 was also implicated in the activation of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) signaling pathways. The findings presented in this study are crucial for a comprehensive understanding of the real pathogenicity of SLS-producing β-hemolytic AGS in the latest clinical situations.

Introduction

Anginosus group streptococci (AGS) have historically been recognized as opportunistic pathogens inhabiting the oral cavity in humans. Their virulence has been considered less significant than that of typical human pathogenic streptococci, such as *Streptococcus pyogenes* (commonly known as Group A streptococci or GAS), *Streptococcus agalactiae* (Group B streptococci or GBS), and *Streptococcus pneumoniae* (pneumococci). Recent studies have demonstrated the pathogenic potential of AGS in humans, including abscess formation and ectopic infections Cervera-Hernandez and Pohl (2017); Rawla et al. (2017); Finn et al. (2018); Morii et al. (2018); Pilarczyk-Zurek et al. (2022). Some strains belonging to AGS exhibit obvious β-hemolysis on blood agar plate cultures, and these β-hemolytic strains have been reported to produce two types of β-hemolysins as virulence factors. One of these hemolysins is intermedilysin (ILY), a protein hemolysin with

Abbreviations: AGS, Anginosus group streptococci; BHI, brain heart infusion; CCK-8, Cell Counting Kit-8; CDC, cholesterol-dependent cytolysin; EGTA, *O,O*'-bis(2-aminoethyl)ethyleneglycol-*N,N,N*',*N*'-tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GAS, Group A streptococci; GBS, Group B streptococci; GCF, gingival crevicular fluid; HSA, human serum albumin; ILY, intermedilysin; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PLY, pneumolysin; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SAA, *Streptococcus anginosus* subsp. *anginosus*; SLO, streptolysin O; SLS, streptolysin S; TMB, 3,3',5,5'-tetramethylbenzidine; TOMMs, thiazole and oxazole-modified microsins; VLY, vaginolysin.

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human-specific action categorized as a cholesterol-dependent cytolysin (CDC) secreted by *S. intermedius* Nagamune et al. (1996); Sukeno et al. (2005). The other β-hemolysin is streptolysin S (SLS), a peptide hemolysin secreted by β-hemolytic subgroups of AGS, excluding *S. intermedius*, namely *S. anginosus* and *S. constellatus* Tabata et al. (2013), Tabata et al. (2014), Asam et al. (2013). SLS is produced as the transcribed product of an operon composed of nine genes, from *sagA* to *sagI*, commonly referred to as the "*sag* operon" Nizet et al. (2000). The formation of heterocyclic rings (thiazole and oxazole) in the precursor peptide which is the transcribed product of *sagA* is reported to be essential for the hemolytic activity of SLS Mitchell et al. (2009). Thus, SLS is one of the bacteriocins called "thiazole- and oxazole-modified microsins (TOMMs)" Melby et al. (2011); Cox et al. (2015). Interestingly, the *sag* operon for β-hemolytic strains of *S. anginosus* subsp. *anginosus* (SAA) is composed of 10 genes, including two *sagA* genes named *sagA1* and *sagA2*, and the remaining genes *sagB* to *sagI* Tabata et al. (2013); Asam et al. (2013). Owing to these genetic characteristics, β-hemolytic SAA (β-SAA) produces two functional mature SLSs with different amino acids Tabata et al. (2013); Asam et al. (2013). Including the SLSs secreted from β-SAA, SLS secreted by β-hemolytic AGS strains was reported to function not only as a hemolysin but also as a cytolysin in cultured human cell lines Tabata et al. (2014), (2019).

The SLS produced by streptococci of the pyogenic group has been reported to exhibit hemolytic activity and cytotoxicity both *in vitro* and *in vivo* Betschel et al. (1998); Miyoshi-Akiyama et al. (2005); Sumitomo et al. (2011); Flaherty et al. (2015); Higashi et al. (2016); Hammers et al. (2022). For instance, SLS produced by *S. pyogenes* enhances the signaling pathway for proinflammatory cytokine responses in human keratinocytes during GAS infection Flaherty et al. (2018). Additionally, SLS has been shown to directly activate nociceptor neurons, inducing pain during infection Pinho-Ribeiro et al. (2018). In mouse models, SLS from *S. pyogenes* contributes to the induction of necrotic lesions during the early stages of infection *in vivo* Fontaine et al. (2003). Recently, our research expanded on existing knowledge, revealing that SLS secreted by β-SAA and other SLS-producing streptococci, including *S. pyogenes*, is stabilized in the presence of human serum albumin (HSA), resulting in enhanced hemolysis Yokohata et al. (2023). Furthermore, HSA-stabilized SLS was found to be cytotoxic to the human oral squamous cell carcinoma cell line HSC-2. We demonstrated the contribution of enhanced Ca^{2+}-influx-dependent proinflammatory signaling to the cytotoxicity of SLS in HSC-2 Yamada et al. (2023). At lesion sites such as gingivitis and periodontal disease, it has been suggested that SLS secreted by hemolytic human opportunistic streptococci, including SAA, is stabilized in the presence of HSA. Moreover, in the presence of HSA, cytotoxicity is induced by SLS secreted at ectopic infection sites, establishing the SLS-dependent pathogenicity of β-SAA. To gain accurate insights into the potential pathogenesis of SLS-dependent ectopic infections by human opportunistic β-hemolytic AGS, including β-SAA, and SLS-producing pyogenic group streptococci such as *S. pyogenes,* an investigation into the SLS-dependent response of the human host is necessary.

In this study, our objective was to uncover the potential SLS-dependent pathogenicity of β-SAA, β-hemolytic AGS, and SLS-producing pyogenic group streptococcal species/strains during ectopic infections in the bloodstream. We focused on examining the subsequent immune responses in the human acute monocytic leukemia cell line THP-1, with a specific emphasis on the production of proinflammatory cytokines in response to SLS action.

Materials and methods

Bacterial strains and culture conditions

In this study, we utilized the type strain of SAA, strain NCTC10713^T, which exhibits SLS-dependent β-hemolysis. Additionally, a non-β-hemolytic mutant derived from NCTC10713^T, lacking both *sagA1* and *sagA2* genes (Δ*sagA*s) (Tabata et al., 2013), was employed. These strains underwent pre-incubation in brain heart infusion (BHI) broth (Becton Dickinson and Company, Franklin Lakes, NJ, USA) at 37 °C overnight in a 5 % CO_2 atmosphere. Following preincubation, the bacteria were inoculated into the co-cultivation medium [RPMI-1640 medium (FUJIFILM Wako, Osaka, Osaka, Japan) containing 1.0 % (w/v) of recombinant human serum albumin (HSA; No. 19597–14, Nacalai Tesque Inc., Kyoto, Kyoto, Japan), 10 % (v/v) of BHI broth, 25 mM HEPES (pH 7.4)] to an optical density at 600 nm (OD_{600}) of 0.01 and then incubated for 4 h at 37 °C in a 5 % CO_2 atmosphere. The bacteria-free supernatant was obtained by centrifugation (13,000 × *g*, 5 min, RT), and an antibiotic mixture stock solution (FUJIFILM Wako) was added to inhibit bacterial growth (final concentration: 200 U/mL penicillin G and 200 μg/mL streptomycin). Microscopic observation confirmed no bacterial growth during the preparation of the cell culture supernatant for the downstream assay.

Human cell line and culture condition

The human acute monocytic leukemia cell line THP-1 (RCB1189; RIKEN BRC, Ibaraki, Tsukuba, Japan) was cultured in RPMI-1640 medium (FUJIFILM Wako) containing 10 % (v/v) heat-inactivated fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin G and 100 μg/mL streptomycin; FUJIFILM Wako) at 37 °C in a 5 % CO_2 atmosphere. For each assay, THP-1 cells were resuspended in fresh RPMI-1640 medium and seeded onto plates at an appropriate cell density. As needed, THP-1 cells were treated with the prepared bacterial culture supernatant containing 2.5 mM *O,O*'-bis(2-aminoethyl)ethyleneglycol-*N,N,N*',*N*'-tetraacetic acid (EGTA) to investigate the contribution of Ca^{2+} influx.

Cytotoxicity assay

The cytotoxicity of the prepared bacterial culture supernatant containing SLS on THP-1 cells was assessed using the CellTox Green Cytotoxicity Assay (Promega, Madison, WI, USA) and the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan), according to the manufacturer's protocols. In the CellTox Green Cytotoxicity Assay, THP-1 cell suspension (1.0 × 10^6 cells/mL) was dispensed at 50 μL/well into a clear-bottom 96-well black plate. The 50 μL of prepared bacterial culture supernatant was added to each well (final cell density: 0.5 × 10^5 cells/100 μL/well) and incubated for 1, 2, and 4 h. The viability of the cells incubated in the bacterial culture supernatants was evaluated according to the manufacturer's instructions. The fluorescence of the samples was measured using a plate reader (Infinite® 200 PRO M Nano+; TECAN, Männedorf, Zürich, Switzerland) with excitation and emission wavelengths at 490 nm and 525 nm, respectively.

For the CCK-8 assay, THP-1 cell suspension (1.0 × 10^6 cells/mL) was dispensed at 50 μL/well into 96-well plate. The 50 μL of prepared bacterial culture supernatant was added to each well (final cell density: 0.5 × 10^5 cells/100 μL/well) and incubated for 4, 8, and 12 h. After the incubation period, 10 μL/well of CCK-8 reagent was added, followed by further incubation for 1 h at 37 °C in a 5 % CO_2 atmosphere. The absorbance at 450 nm was measured using a plate reader (Infinite® 200 PRO M Nano+) with a reference wavelength at 600 nm. To prepare the background control for this assay, THP-1 cells were treated with Lysis Solution (Promega) and incubated for a few minutes.

Measurement of intracellular Ca^{2+} concentration

The intracellular Ca^{2+} concentration was measured using a Calcium Kit II - Fluo 4 (Dojindo), following the manufacturer's instructions. Briefly, THP-1 cell suspension (2.0 × 10^6 cells/mL) was dispensed at 50 μL/well into a clear-bottom 96-well black plate. Subsequently, 50 μL of Fluo 4-AM solution, prepared with the loading buffer containing Quenching Buffer from the kit, was added and incubated for 1 h at 37 °C in a 5 % CO_2 atmosphere to load the Fluo 4 dye into the cells. The

prepared bacterial culture supernatant (100 μL /well) was then added, and the time-course fluorescence was measured (final cell density: 1.0×10^5 cells/200 μL/well). The dynamic change in fluorescence intensity was monitored at 5-s intervals using a plate reader (Infinite® 200 PRO M Nano+) with excitation and emission wavelengths at 490 nm and 518 nm, respectively. THP-1 cells treated with 0.1 μM ionomycin were also prepared as positive controls.

RNA isolation and reverse transcription quantitative polymerase chain reaction (RT-qPCR)

THP-1 cell suspension (1.0×10^6 cells/mL) was dispensed into 12-well plates at 750 μL and treated with the same volume (750 μL) of prepared bacterial culture supernatant for 4 h at 37 °C in a 5 % CO_2 atmosphere (final cell density: 7.5×10^5 cells/1.5 mL/well). After washing with PBS and centrifugation (200 × *g*, 5 min, RT) to collect the cells, total RNA was extracted using the NucleoSpin® RNA Plus kit (TaKaRa Bio Inc., Kusatsu, Shiga, Japan), following the manufacturer's protocol. cDNA was synthesized from 2 μg of total RNA using a High-Capacity cDNA Reverse Transcription Kit with an RNase inhibitor (Thermo Fisher Scientific, Waltham, MA, USA), as per the manufacturer's instructions. For reverse transcription, the oligo $(dT)_{15}$ primer (TaKaRa Bio Inc.) was used in addition to random primers supplied with the kit. Quantitative real-time PCR was performed on a Thermal Cycler Dice® Real Time PCR System *Lite* TP700 (TaKaRa Bio Inc.) using the comparative Ct method (ΔΔCt) with TB Green® *Premix Ex Taq* II Tli RNase H Plus (TaKaRa Bio Inc.). All Ct values for the target gene expression were normalized to those of GAPDH. The primers used in this study are listed in Table 1.

Enzyme-linked immunosorbent assay (ELISA)

The production of IL-8 was quantitatively measured using a Human IL-8/CXCL8 DuoSet® ELISA kit (R&D Systems, Minneapolis, Minnesota, USA), according to the manufacturer's protocol. THP-1 cell suspension (1.0×10^6 cells/mL) was dispensed into 12-well plates at 750 μL and treated with the same volume (750 μL) of the prepared bacterial culture supernatant for 12 h (final cell density: 7.5×10^5 cells/1.5 mL/well). After incubation, the culture supernatant of the cells was collected by centrifugation (13,000 × *g*, 5 min, 4 °C). The ELISA was performed according to the manufacturer's instructions. Briefly, a clear-bottomed 96-well plate (high binding) was coated with a Human IL-8 capture antibody and incubated overnight at room temperature. The plate was washed and blocked for 1 h at room temperature (15–20 °C). The prepared culture supernatant samples and standards were added, and the cells were incubated for 2 h at room temperature. Human IL-8 Detection antibody was added and incubated for another 2 h at room temperature. Subsequently, streptavidin-HRP solution was added, and the mixture was incubated for 20 min at room temperature. After a final wash, 3,3′,5,5′-tetramethylbenzidine (TMB) Microwell Peroxidase Substrate (SeraCare, Milford, Massachusetts, USA) was added to the wells and incubated at room temperature. After the development of an adequate color, phosphoric acid was added to stop the reaction. The plate was read using a plate reader (Infinite® 200 PRO M Nano+) at the absorbance of 450 nm with a reference wavelength of 600 nm.

Table 1
Primers for RT-qPCR used in this study.

Primer name	Primer sequence (5′–3′)	Size (bp)	Tm (°C)
IL-6_forward	ATGAACTCCTTCTCCACAAGCGC	23	56.9
IL-6_reverse	GAAGAGCCCTCAGGCTGGACTG	22	60.3
TNF-α_forward	TCCACCCATGTGCTCCTCAC	20	52.0
TNF-α_reverse	TCTGGCAGGGGCTCTTGATG	20	52.0
CCL3_forward	ATGCAGGTCTCCACTGCTGC	20	55.8
CCL3_reverse	TTCTGGACCCACTCCTCACTG	21	56.2
CCL4_forward	CCTCATGCTAGTAGCTGCCTT	21	50.5
CCL4_reverse	CAGTTCCAGGTCATACACGTACTC	24	53.6
CXCL8_forward	ATGACTTCCAAGCTGGCCGTGCT	23	58.7
CXCL8_reverse	TCTCAGCCCTCTTCAAAAACTTCTC	25	55.9
GAPDH_forward	GTCTTCACCACCATGGAGAAGGCT	24	55.3
GAPDH_reverse	CATGCCAGTGAGCTTCCCGTTCA	23	55.0

Treatment of kinase inhibitors

The MAPK kinase inhibitors PD98059 (Millipore, Burlington, Massachusetts, USA), SB203580 (Millipore), and SP600125 (Millipore) were used to inhibit the ERK1/2, p38MAPK, and c-Jun N-terminal kinase (JNK) signaling pathways, respectively. Each inhibitor was added to a final concentration of 10 μM. THP-1 cell suspension (2.0×10^6 cells/mL) was dispensed at 750 μL/well into 12-well plate and pretreated with each inhibitor for 2 h at 37 °C in a 5 % CO_2 atmosphere then the THP-1 cells were treated with the same volume (750 μL) of prepared bacterial culture supernatant for 4 h (RT-qPCR) or 12 h (ELISA) at 37 °C in a 5 % CO_2 atmosphere (final cell density: 7.5×10^5 cells/1.5 mL/well). As a control, the cells were treated with 0.1 % DMSO instead of the inhibitor.

Statistical analysis

Statistical evaluation was performed using R software (version 4.3.2, https://www.r-project.org/). Differences between groups were calculated using an unpaired *t*-test (Student's *t*-test or Welch's *t*-test). The threshold for statistical significance was set at a p-value < 0.05.

Results

SLS-dependent cytotoxicity to THP-1 cells

In a previous report, it was noted that the culture supernatant of the SLS-producing SAA strain NCTC10713^T induces hemolysis and cytotoxicity in human oral squamous cell carcinoma HSC-2 Tabata et al. (2019). To assess the cytotoxicity of the culture supernatant prepared from the NCTC10713^T strain against immune cell lines, both the CellTox Green Cytotoxicity Assay and the CCK-8 assay were conducted on the human monocytic leukemia cell line THP-1.

The results of the CellTox Green Cytotoxicity Assay (Fig. 1A), which evaluates cellular membrane damage, demonstrated a significant increase in fluorescence intensity, indicating membrane damage in THP-1 cells incubated with the culture supernatants of NCTC10713^T after only 1 h. In contrast, THP-1 cells incubated with the culture supernatants of the strain Δ*sagA*s showed no significant cytotoxicity for at least 4 h of incubation.

Additionally, the results of the CCK-8 assay (Fig. 1B), measuring cellular metabolic activity, revealed a significant decrease in the viability of THP-1 cells treated with the culture supernatants of the NCTC10713^T strain for 8 h. Conversely, no significant decrease in viability was observed in THP-1 cells incubated with the culture supernatant of the non-hemolytic mutant strain Δ*sagA*s for the same incubation time. After 12 h of incubation, a decrease in cell viability was observed for both culture supernatants (strains NCTC10713^T and Δ*sagA*s); however, greater cytotoxicity was evident in THP-1 cells incubated with the culture supernatant of strain NCTC10713^T, demonstrating SLS-dependent hemolysis/cytolysis (Fig. 1B). These findings suggest that SLS secreted from β-SAA strains induces significant cytotoxicity due to SLS-dependent cellular membrane damage, resulting in decreased cell viability in THP-1 cells.

SLS-dependent influx of Ca^{2+} in THP-1 cells

Intracellular Ca^{2+} fluctuations were examined in THP-1 cells incubated with the culture supernatant using a Calcium Kit II - Fluo 4 (Dojindo) to investigate SLS-induced intracellular Ca^{2+} influx. As depicted in Fig. 2A, an increase in intracellular Ca^{2+} was observed in THP-1 cells incubated with the culture supernatant of NCTC10713^T. In

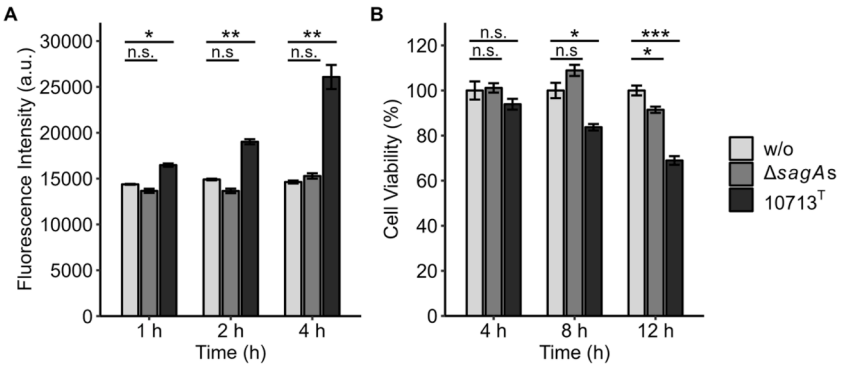

Fig. 1. SLS-dependent cytotoxicity to THP-1 cells.
THP-1 cells were incubated with the culture supernatant prepared from *S. anginosus* subsp. *anginosus* strain NCTC10713^T (black bars) or its *sagA*-genes (*sagA1* and *sagA2*) deletion mutant (Δ*sagA*s) (dark-gray bars) at specified times. The cell viability was determined by CellTox Green assay (Promega) (A) and by CCK-8 assay (Dojindo) (B). The light-grey bars indicate THP-1 cells incubated in the medium without co-cultivation of the tested strains as the control. Triplicate samples were assayed, and the result is presented as mean ± SD. The significance of the differences in SLS-dependent cytotoxicity was evaluated using Student's *t*-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n.s. not significant). a.u.: arbitrary unit.

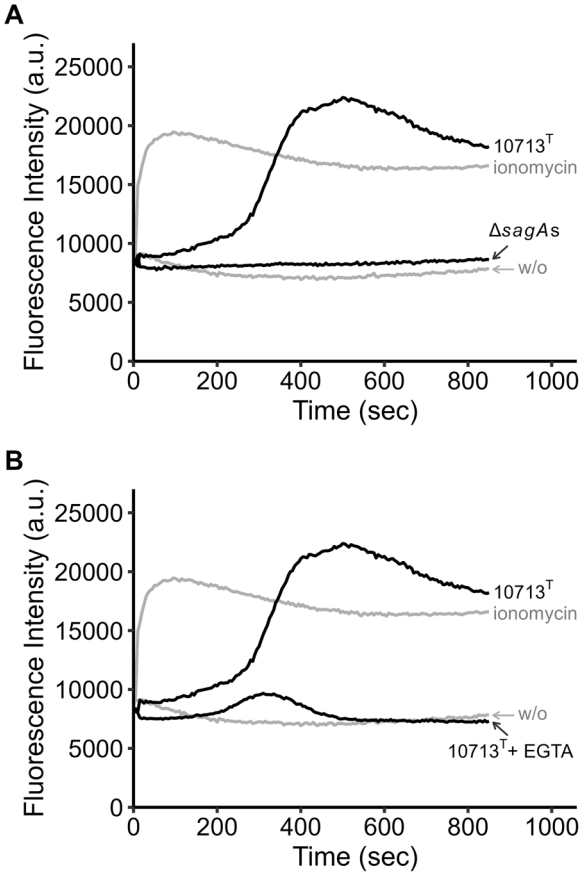

Fig. 2. SLS-dependent influx of extracellular Ca^{2+} into THP-1 cells.
The influx of Ca^{2+}into THP-1 cells incubated with the culture supernatant prepared from the tested strains was evaluated using a Calcium Kit II - Fluo 4 (Dojindo). THP-1 cells treated with 0.1 μM ionomycin were used as Ca^{2+}-permeabilized controls. The "w/o" indicates THP-1 cells incubated in the medium without co-cultivation of the tested strains as the control. This is the representative result from total of four technical replicates derived from two of biological replicates of the assay.

contrast, no increase in intracellular Ca^{2+} levels was observed for the cells incubated with the culture supernatant of Δ*sagA*s, nearly matching the levels in THP-1 cells incubated with the medium only (background control). Furthermore, the increase in intracellular Ca^{2+} in THP-1 cells incubated with the culture supernatant of strain NCTC10713^T was significantly inhibited by extracellular Ca^{2+} chelation with EGTA (Fig. 2B). These results indicate that SLS secreted from the SAA strain NCTC10713^T induces SLS-dependent extracellular Ca^{2+} influx in THP-1 cells.

SLS-dependent expression of cytokine-encoding genes in THP-1 cells

The SLS-dependent expression of genes encoding proinflammatory cytokines in THP-1 cells incubated with culture supernatants prepared from NCTC10713^T and Δ*sagA*s was determined using quantitative real-time polymerase chain reaction (RT-qPCR). As a result, the expression levels of genes encoding IL-6, TNF-α, CCL3, CCL4, and IL-8/CXCL8 were significantly up-regulated in cells incubated with the culture supernatant of NCTC10713^T for 4 h (Fig. 3). Among these, the gene encoding IL-8 showed the highest increase in relative expression levels. To confirm the increased expression of IL-8 as the polypeptide product, the culture supernatants of THP-1 cells incubated with bacterial culture supernatants were assessed by ELISA to determine IL-8 production. As shown in Fig. 4, IL-8 secretion increased in a time-dependent manner from 4 to 12 h of incubation with the SLS-containing culture supernatant prepared from strain NCTC10713^T. In contrast, there was no significant increase in IL-8 secretion in THP-1 cells incubated with the culture supernatants of Δ*sagA*s compared to cells incubated with the medium without co-cultivation of the tested strains.

The SLS-dependent upregulation of the expression of the five genes encoding the cytokines described above was significantly suppressed in cells in the presence of the Ca^{2+}-specific chelator EGTA (Fig. 3). Additionally, the secretion of the IL-8 polypeptide after incubation with SLS-containing culture supernatant prepared from strain NCTC10713^T was also significantly decreased (Fig. 4). These results suggest that the SLS-dependent increase in cytokine gene expression was caused by Ca^{2+} influx into THP-1 cells following SLS-induced cellular membrane damage.

Effects of MAPK inhibitors on SLS-dependent cytokine expression in THP-1 cells

To further elucidate the molecular mechanisms underlying SLS-dependent cytokine expression in THP-1 cells, the effects of MAPK inhibitors [PD98059 (ERK1/2 inhibitor), SB203580 (p38 inhibitor), and SP600125 (JNK inhibitor)] on cytokine expression were investigated. THP-1 cells were pre-treated with the respective MAPK inhibitors for 2 h and incubated with the prepared bacterial culture supernatant of strain NCTC10713^T for 4 h. After incubation, the expression of cytokine genes

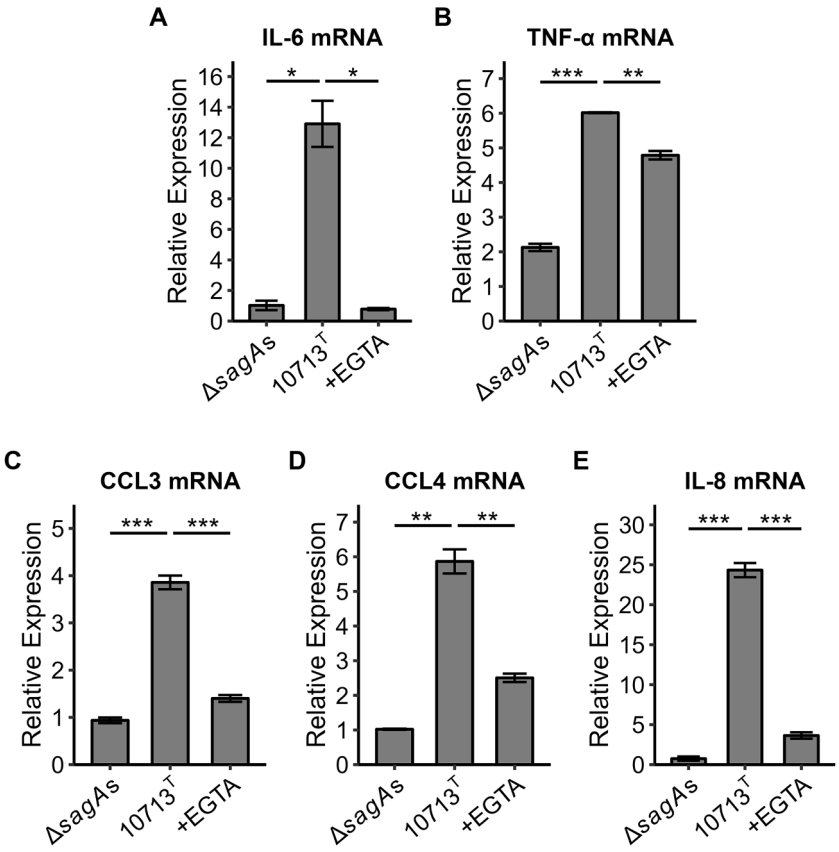

Fig. 3. SLS-dependent and Ca^{2+}-influx-induced relative expression of cytokine-encoding genes in THP-1 cells.
The SLS- and Ca^{2+}-influx-dependent gene expressions for proinflammatory cytokines encoding IL-6 (A) and TNF-α (B), and chemokines [CCL3/MIP-1α (C), CCL4/MIP-1β (D), and IL-8/CXCL8 (E)] were examined. THP-1 cells were incubated with the culture supernatant prepared from *S. anginosus* subsp. *anginosus* strain NCTC10713^T or its *sagA*-genes (*sagA1* and *sagA2*) deletion mutant (Δ*sagAs*) for 4 h. The contribution of Ca^{2+} influx to gene expression was evaluated in the presence of EGTA. The results were evaluated as relative expression against THP-1 cells incubated in the medium without co-cultivation of the tested strains. Triplicate samples were assayed, and the result is presented as mean ± SD. The significance of differences was evaluated using Student's *t*-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

was determined using RT-qPCR. For IL-8, the production of IL-8 in the culture supernatant of THP-1 cells was also measured using ELISA. The results showed that THP-1 cells pre-treated with PD98059 significantly suppressed the SLS-dependent up-regulation of the genes encoding TNF-α, CCL3, and IL-8 compared to cells untreated with the inhibitor (Figs. 5B, 5C, and 5E). Pretreatment with SB203580 significantly suppressed the upregulation of genes encoding IL-6, CCL3, CCL4, and IL-8 (Figs. 5A, 5C, 5D, and 5E). The upregulation of genes encoding CCL3 and CCL4 was also significantly suppressed by pretreatment with SP600125 (Figs. 5C and 5D). Furthermore, SLS-dependent IL-8 secretion into the culture supernatant was significantly decreased in THP-1 cells pre-treated with PD98059 or SB203580 (Fig. 6), as expected from the results of gene expression by RT-qPCR. These results suggest that SLS-dependent cytokine expression is regulated by MAPKs, and both the ERK and p38 pathways are especially important for the response to SLS in THP-1 cells.

Discussion

S. anginosus has traditionally been identified as an opportunistic pathogen inhabiting the human oral cavity. Its pathogenicity is considered less significant than that of human pathogenic streptococci, such as Group A streptococci (*S. pyogenes*) and *Streptococcus pneumoniae*. However, recent reports have increasingly highlighted the relevance of the pathogenicity of AGS strains in humans. Interestingly, this species has frequently been isolated from infections and disorders outside their normal oral cavity residence (Pilarczyk-Zurek et al., 2022), making it a potential causative bacterium for ectopic infections in humans. Among the representative human pathogenic streptococci, both *S. pyogenes* and *S. pneumoniae* produce the β-hemolysin belonging to the CDC named streptolysin O (SLO) and pneumolysin (PLY), respectively. In addition to CDC, *S. pyogenes* also produces another β-hemolysin, SLS. On the other hand, except for *S. intermedius* which produces ILY, streptococcal species belonging to AGS produce SLS as a sole β-hemolytic factor Tabata and Nagamune. (2021). However, the detailed and enough investigation to understand the contribution of SLS to the pathogenicity of β-hemolytic SLS-producing AGS has not yet been conducted.

SLS is a peptide-type β-hemolysin generally produced by pathogenic pyogenic group streptococci such as *S. pyogenes* and has demonstrated cytotoxicity both *in vitro* and *in vivo*. For instance, SLS produced by *S. pyogenes* enhances the signaling pathway of the proinflammatory cytokine response in human keratinocytes during infection, directly activates nociceptor neurons, and induces pain during infection Pinho-Ribeiro et al. (2018). Moreover, SLS contributes to the induction of necrotic lesions during the early stages of infection in mouse models *in vivo* Fontaine et al. (2003). According to the results presented in these reports, SLS produced by *S. pyogenes* is increasingly recognized as a significant pathogenic factor contributing to disorders and diseases. Notably, we previously reported that β-hemolytic strains belonging to AGS, excluding *S. intermedius*, also produce SLS, which functions not only as β-hemolysin but also as a cytolysin Tabata et al. (2014) (2019). The nucleotide sequence of *sagA1* and *sagA2* of β-hemolytic SAA shows high identity with the *sagA* gene of *S. pyogenes* and the transcribed products also show higher amino acid sequence similarity/identity; the

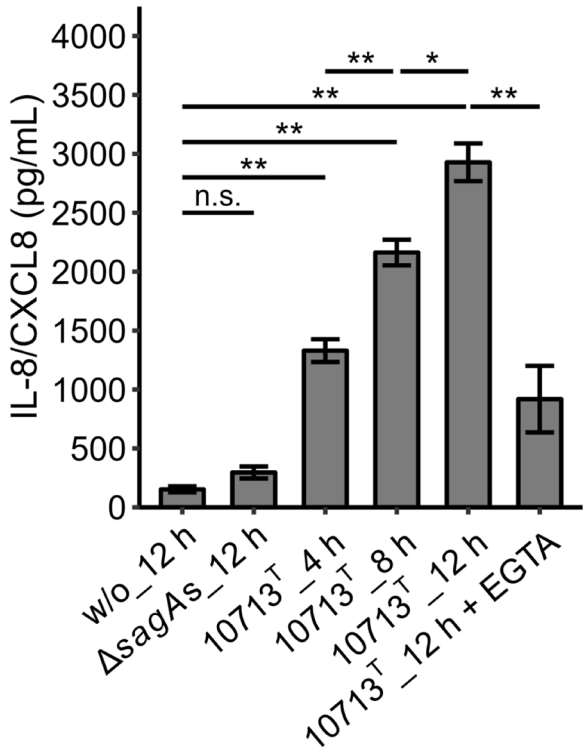

Fig. 4. SLS-dependent IL-8/CXCL8 secretion from THP-1 cells.
THP-1 cells were incubated with the culture supernatant prepared from *S. anginosus* subsp. *anginosus* strain NCTC10713^T or its *sagA*-genes (*sagA1* and *sagA2*) deletion mutant (Δ*sagAs*) at the specified times. After incubation, the culture supernatant was collected, and the concentration of IL-8 was determined by ELISA. The contribution of Ca^{2+} influx to the production of IL-8 was also evaluated in the presence of EGTA. The "w/o" indicates THP-1 cells incubated in the medium without co-cultivation of the tested strains as the control. Triplicate samples were assayed, and the result is presented as mean ± SD. The significance of the differences was evaluated using Student's *t*-test (*$p < 0.05$, **$p < 0.01$, n.s. not significant).

similarity/identity of the transcriptional product of both *sagA1* and *sagA2* to the SLS of *S. pyogenes* is 92 %/68 % and 92 %/72 %, respectively. However, some differences in the sequence were observed in the 3′-region of *sagA* genes Tabata et al. (2013). The information suggests that the transcriptional products from both *sagA1* and *sagA2* genes of β-hemolytic SAA may have some different function to the SLS produced from *S. pyogenes*. However, the detailed mechanisms of the cytotoxicity of the SLSs produced by β-hemolytic SAA in human cells and the contribution to the pathogenicity of humans remain unclear.

Recently, we reported that the SLS secreted by β-hemolytic subgroup strains belonging to AGS and other SLS-producing streptococci, including *S. pyogenes*, is stabilized in the presence of HSA and enhances hemolysis Yokohata et al. (2023). Furthermore, HSA-stabilized SLS was found to be cytotoxic to the human oral squamous cell carcinoma cell line HSC-2. We described the Ca^{2+} influx-dependent enhanced expression of immediate early genes and genes encoding cytokines following SLS-dependent cytotoxicity in HSC-2 Yamada et al. (2023). Since serum albumin is present in gingival crevicular fluid (GCF) (Carneiro et al., 2014), the stabilization of SLS by HSA may contribute to the exacerbation of disorders such as gingivitis and periodontal disease. Additionally, as HSA is the most abundant protein in human plasma, it is suggested that if SLS-producing AGS strains are translocated into the bloodstream, the secreted SLS from the strains will be effectively stabilized, and SLS-dependent cytotoxicity may be induced at the site of ectopic infection from their resident oral cavity.

When SLS-producing streptococci translocate into the bloodstream, it is hypothesized that SLS-dependent cellular damage occurs to vascular endothelial cells and immune cells during the circulation of translocated streptococci, reaching the site of infection. Macrophages, crucial in protecting against pathogen invasion, differentiate from monocytes. They play a pivotal role in the early stages of infection by eliminating pathogens through phagocytosis, an essential mechanism for host defense against microbial infections Lendeckel et al. (2022). Macrophages also initiate the inflammatory response by releasing cytokines, including chemokines, which attract other immune cells to the site of inflammation Lendeckel et al. (2022). Therefore, it is essential to investigate the cellular response of immune cells, such as monocytes and macrophages, to SLS produced by β-hemolytic streptococcal strains, which have the potential to translocate into the bloodstream from the oral cavity.

In this study, we investigated the SLS-dependent cellular response of the human acute monocytic leukemia cell line THP-1 to unveil the pathogenic potential of β-SAA and the contribution of SLS produced by them. The findings from this study are summarized in Table 2. The results demonstrated that SLS secreted by the β-SAA strain NCTC10713^T and stabilized in the presence of HSA exhibited cytotoxicity towards THP-1 cells (Fig. 1) and induced Ca^{2+} influx into THP-1 cells (Fig. 2). Additionally, HSA-stabilized SLS enhanced the expression of genes encoding proinflammatory cytokines (Fig. 3) and the production of IL-8 (Fig. 4) in a Ca^{2+}-dependent manner. Ca^{2+} regulates various cellular functions by controlling signaling pathways in eukaryotic cells. In the context of infectious diseases, previous studies have reported that Ca^{2+} induces cytokine production, such as IL-6 and IL-8, during *Escherichia coli*-induced renal inflammation and IL-8 in proinflammation during Pneumocystis pneumonia infection Uhlén et al. (2000); Carmona et al. (2010).

Moreover, our previous findings indicated an increased expression of genes encoding cytokines (IL-6 and IL-8/CXCL8) in HSC-2 cells in an SLS-dependent manner Yamada et al. (2023). These cytokines are vital for infection and immune responses as IL-6 is a classic proinflammatory cytokine crucial in inflammation, and IL-8 is a representative chemokine promoting the migration of immune cells to the site of inflammation Yoshimura (2015); Uciechowski and Dempke (2020). In the present study, using the human acute monocytic leukemia cell line THP-1, enhanced SLS-dependent expression was observed for genes encoding CCL3 and CCL4, in addition to the genes encoding IL-6 and IL-8. Similar to IL-8/CXCL8, both CCL3 and CCL4 function as chemokines. Chemokines are classified into major subfamilies, including CC and CXC chemokines. Typically, CC chemokines exhibit monocyte and lymphocyte chemotaxis and are involved in wound healing and hematopoiesis in the early stages of inflammation, whereas CXC chemokines are involved in neutrophil chemotaxis Liu et al. (2016); Legler and Thelen (2016); Mohammadi and Kariminik (2021). Assuming that IL-8 is produced in blood vessels, it has been shown to be chemotactic and mitogenic against T lymphocytes and smooth muscle cells (Larsen et al., 1989), inducing endothelial cell proliferation (Koch et al., 1992), and angiogenesis Simonini et al. (2000). Furthermore, it has been reported that proinflammatory cytokines and chemokines such as IL-1, IL-6, IL-8, TNF-α, and MCP-1 are released during *Streptococcus suis* infection, promoting inflammation Segura et al. (2002). The cytokines examined in this study are crucial factors for inflammation.

The enhanced expression of genes encoding cytokines, dependent on SLS, was suppressed in the presence of MAPK inhibitors, combined with a specific gene-MAPK inhibitor (Fig. 5). These results indicate that, although the cellular response of THP-1 cells to SLS is initiated by the influx of Ca^{2+} (Fig. 2), the downstream mechanism/pathway differs and is specific for each cytokine-encoding gene regulated by MAPKs. Among the cytokines investigated in this study, a quantitative evaluation was performed for the representative chemokine IL-8 using ELISA. Reflecting the gene-expression results in the presence of the MAPK inhibitor (Fig. 5E), SLS-induced IL-8 secretion into the culture supernatant significantly decreased in THP-1 cells pre-treated with PD98059 (ERK inhibitor) or SB203580 (p38 inhibitor) (see Fig. 6). These findings suggest that SLS-dependent gene expression and IL-8 production are

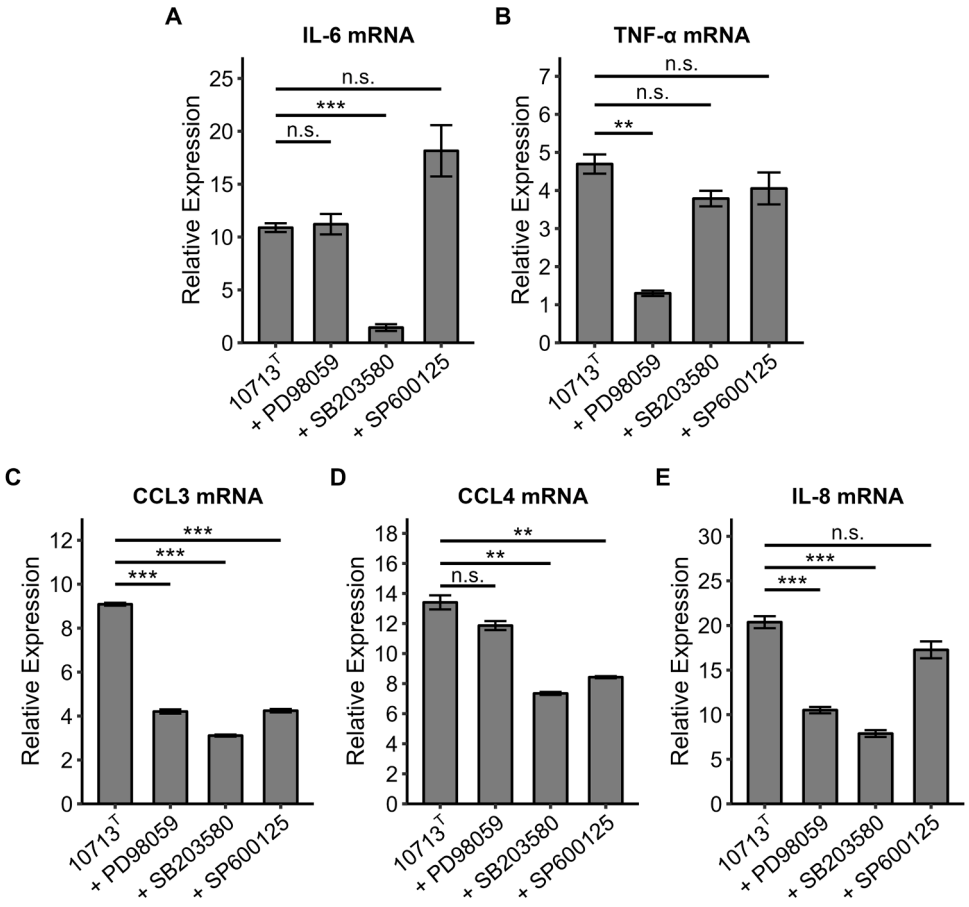

Fig. 5. Effects of MAPK inhibitors to the SLS-dependent expression of cytokine-encoding genes in THP-1.
SLS-dependent gene expression of proinflammatory cytokines encoding IL-6 (A) and TNF-α (B), and chemokines [CCL3/MIP-1α (C), CCL4/MIP-1β (D), and IL-8/CXCL8 (E)] were evaluated. THP-1 cells were pre-treated with each of the MAPK inhibitors (PD98059, SB203580, and SP600125) for 2 h and incubated with the culture supernatant prepared from *S. anginosus* subsp. *anginosus* strain NCTC10713^T for 4 h. These results are presented as the relative expression against THP-1 cells incubated in the medium without co-cultivation of the tested strains. Triplicate samples were assayed, and the result is presented as mean ± SD. The significance of the differences was evaluated using Student's *t*-test (**$p < 0.01$, ***$p < 0.001$, n.s. not significant).

regulated by both the ERK and p38 pathways in THP-1 cells. Intracellular kinases respond to various extracellular stimuli and regulate signaling pathways contributing to cellular processes such as proliferation, differentiation, apoptosis, immune response, and inflammation. Among these pathways, MAPK signaling has been widely reported as a potential target for proinflammatory responses Kyriakis and Avruch (2001). An example of cellular response to a bacterial toxin is vaginolysin (VLY), a protein hemolysin belonging to the cholesterol-dependent cytolysin family produced by *Gardnerella vaginalis* associated with bacterial vaginosis of human; VLY-dependent activation of p38MAPK and induced IL-8 production in human epithelial cells has been reported Gelber et al. (2008). MAPK family proteins, including ERK1/2 and JNK in addition to p38MAPK, have also been reported to induce cytokine expression in macrophages, both *in vitro* and *in vivo* Luo et al. (2012); Lee et al. (1994). This information further supports the importance of the MAPK signaling pathway activated by the peptide-type β-hemolysin of SLS in elucidating the pathogenic mechanism for SLS-producing streptococci, including human opportunistic AGS strains such as SAA.

In conclusion, the present study showed the SLS-dependent cellular response of human acute monocyte cell line THP-1 (summarized in Table 2). The results shown in this study revealed the cytotoxicity of SLS produced by β-hemolytic SAA and the response in human-derived immune cell line. These results suggested the potential SLS-dependent pathogenicity of β-hemolytic human opportunistic SAA strains belonging to AGS in the condition of ectopic infection such as translocated in the bloodstream. Although the pathogenicity of streptococcal strains belonging to AGS has tended to be underestimated until now, understanding the true pathogenicity of SLS-producing β-hemolytic AGS is crucial because these strains reside in an environment where they can translocate into the bloodstream and may cause ectopic infections. The results shown in this manuscript are culture-cell-based *in vitro* study, and the cellular response to SLS secreted from β-hemolytic SAA may differ from *in vivo* conditions due to the presence of the various biological materials in body fluids, including blood, that may affect the activity of SLS. To overcome this limitation, the infection study of SLS-producing β-hemolytic SAA using mouse models may be one of the effective tools. Further studies are currently underway to elucidate the detailed mechanisms of SLS-dependent pathogenicity of AGS strains, such as SAA, in human disorders related to these microbes.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Atsushi Tabata reports financial support was provided by Japan Society for the Promotion of Science. Kazuto Ohkura reports financial support was provided by Japan Society for the Promotion of Science. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

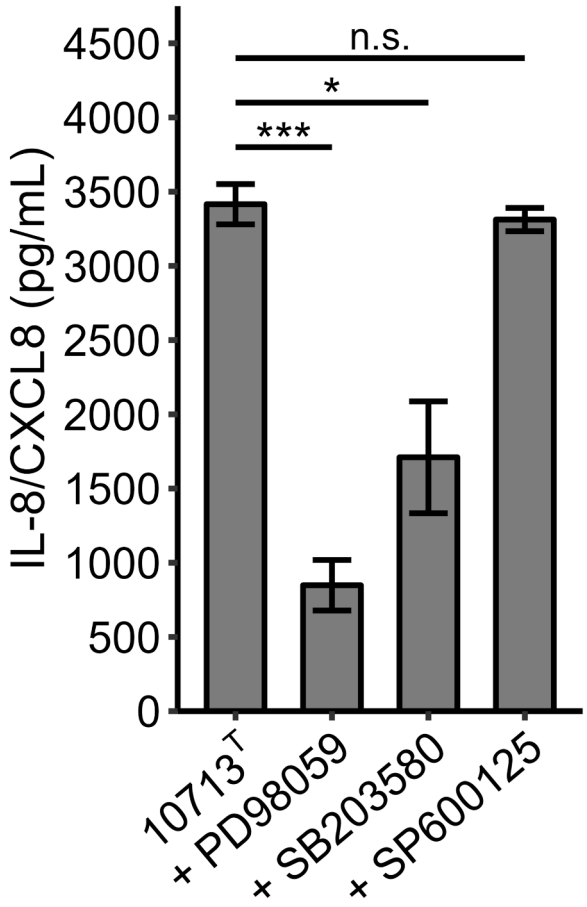

Fig. 6. Effects of MAPK inhibitors on the SLS-dependent IL-8/CXCL8 secretion from THP-1.
THP-1 cells were pre-treated with each of the MAPK inhibitors (PD98059, SB203580, and SP600125) for 2 h and incubated with the culture supernatant prepared from *S. anginosus* subsp. *anginosus* strain NCTC10713^T for an additional 8 h. After incubation, the culture supernatant was collected, and the concentration of IL-8 was determined by ELISA. Triplicate samples were assayed, and the result is presented as mean ± SD. The significance of the differences was evaluated using Student's *t*-test (*$p < 0.05$, ***$p < 0.001$, n.s. not significant).

Table 2
Summary of SLS-dependent response in THP-1.

mRNA	SLS-dependent expression[1)]	Ca^{2+}-dependency[2)]	Regulation by MAPK pathways[3)]		
			ERK	p38	JNK
IL-6	+ +	+ +	−*	+ +	−*
TNF-α	+	+	+ +	−	−
CCL3	+	+ +	+ +	+ +	+ +
CCL4	+	+ +	−	+	+
IL-8	+ +	+ +	+	+ +	−

1) +: The increased expression was less than 10-fold compared to the results of Δ*sagA*s, + +: The increased expression was more than 10-fold compared to the results of Δ*sagA*s.
2) +: The expression was suppressed, and more than half of expression was observed compared to the result of 10713^T, + +: The expression was suppressed, and less than half of expression was observed compared to the result of 10713^T.
3) +: The expression was suppressed, and more than half of expression was observed compared to the result of 10713^T, + +: The expression was suppressed, and less than half of expression was observed compared to the result of 10713^T, −: suppressed expression was not significant, *: gene expression was not suppressed.

Data availability

Data will be made available on request.

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Credit Author Statement

Yugo Yamamori: Validation, Investigation, Writing-Original Draft, Visualization. **Rina Shirai:** Investigation. **Kazuto Ohkura:** Writing-Review & Editing, Funding acquisition. **Hideaki Nagamune:** Writing-Review & Editing, Supervision. **Toshifumi Tomoyasu:** Writing-Review & Editing. **Atsushi Tabata:** Conceptualization, Writing-Original Draft, Writing-Review & Editing, Project administration, Funding acquisition.

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References

Asam, D., Mauerer, S., Walheim, E., Spellerberg, B., 2013. Identification of β-hemolysin-encoding genes in *Streptococcus anginosus*. Mol. Oral. Microbiol. 28, 302–315. https://doi.org/10.1111/omi.12026.

Betschel, S.D., Borgia, S.M., Barg, N.L., Low, D.E., De Azavedo, J.C., 1998. Reduced virulence of group A streptococcal Tn916 mutants that do not produce streptolysin S. Infect. Immun. 66, 1671–1679. https://doi.org/10.1128/IAI.66.4.1671-1679.1998.

Carmona, E.M., Lamont, J.D., Xue, A., Wylam, M., Limper, A.H., 2010. Pneumocystis cell wall beta-glucan stimulates calcium-dependent signaling of IL-8 secretion by human airway epithelial cells. Respir. Res. 11, 95. https://doi.org/10.1186/1465-9921-11-95.

Carneiro, L.G., Nouh, H., Salih, E., 2014. Quantitative gingival crevicular fluid proteome in health and periodontal disease using stable isotope chemistries and mass spectrometry. J. Clin. Periodontol. 41, 733–747. https://doi.org/10.1111/jcpe.12262.

Cervera-Hernandez, M.E., Pohl, D., 2017. Splenic abscess caused by *Streptococcus anginosus* following laparoscopic sleeve gastrectomy: a case report of a rare complication of bariatric surgery. J. Surg. Case Rep. 2017 https://doi.org/10.1093/jscr/rjx072 rjx072.

Cox, C.L., Doroghazi, J.R., Mitchell, D.A., 2015. The genomic landscape of ribosomal peptides containing thiazole and oxazole heterocycles. BMC. Genomics. 16, 778. https://doi.org/10.1186/s12864-015-2008-0.

Finn, T., Schattner, A., Dubin, I., Cohen, R., 2018. *Streptococcus anginosus* endocarditis and multiple liver abscesses in a splenectomised patient. BMJ Case Rep. 2018, bcr2018224266 https://doi.org/10.1136/bcr-2018-224266.

Flaherty, R.A., Puricelli, J.M., Higashi, D.L., Park, C.J., Lee, S.W., 2015. Streptolysin S promotes programmed cell death and enhances inflammatory signaling in epithelial keratinocytes during group a streptococcus infection. Infect. Immun. 83, 4118–4133. https://doi.org/10.1128/IAI.00611-15.

Flaherty, R.A., Donahue, D.L., Carothers, K.E., Ross, J.N., Ploplis, V.A., Castellino, F.J., Lee, S.W., 2018. 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. 8, 211. https://doi.org/10.3389/fcimb.2018.00211.

Fontaine, M.C., Lee, J.J., Kehoe, M.A., 2003. Combined contributions of streptolysin O and streptolysin S to virulence of serotype M5 *Streptococcus pyogenes* strain Manfredo. Infect. Immun. 71, 3857–3865. https://doi.org/10.1128/IAI.71.7.3857-3865.2003.

Gelber, S.E., Aguilar, J.L., Lewis, K.L.T., Ratner, A.J., 2008. Functional and phylogenetic characterization of Vaginolysin, the human-specific cytolysin from *Gardnerella vaginalis*. J. Bacteriol. 190, 3896–3903. https://doi.org/10.1128/JB.01965-07.

Hammers, D.E., Donahue, D.L., Tucker, Z.D., Ashfeld, B.L., Ploplis, V.A., Castellino, F.J., Lee, S.W., 2022. Streptolysin S targets the sodium-bicarbonate cotransporter NBCn1 to induce infammation and cytotoxicity in human keratinocytes during group a streptococcal infection. Front. Cell Infect. Microbiol. 12, 1002230 https://doi.org/10.3389/fcimb.2022.1002230.

Higashi, D.L., Biais, N., Donahue, D.L., Mayfield, J.A., Tessier, C.R., Rodriguez, K., Ashfeld, B.L., Luchetti, J., Ploplis, V.A., Castellino, F.J., Lee, S.W., 2016. Activation

of band 3 mediates group A *Streptococcus* streptolysin S-based beta-haemolysis. Nat. Microbiol. 1, 15004. https://doi.org/10.1038/nmicrobiol.2015.4.
Kyriakis, J.M., Avruch, J., 2001. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol. Rev. 81, 807–869. https://doi.org/10.1152/physrev.2001.81.2.807.
Koch, A.E., Polverini, P.J., Kunkel, S.L., Harlow, L.A., DiPietro, L.A., Elner, V.M., Elner, S. G., Strieter, R.M., 1992. Interleukin-8 as a macrophage-derived mediator of angiogenesis. Science (1979) 258, 1798–1801. https://doi.org/10.1126/science.1281554.
Larsen, C.G., Anderson, A.O., Appella, E., Oppenheim, J.J., Matsushima, K., 1989. The neutrophil-activating protein (NAP-1) is also chemotactic for T lymphocytes. Science (1979) 243, 1464–1466. https://doi.org/10.1126/science.2648569.
Lee, J.C., Laydon, J.T., McDonnell, P.C., Gallagher, T.F., Kumar, S., Green, D., McNulty, D., Blumenthal, M.J., Keys, J.R., Landvatter, S.W., Strickler, J.E., McLaughlin, M.M., Siemens, I.R., Fisher, S.M., Livi, G.P., White, J.R., Adams, J.L., Young, P.R., 1994. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature 372, 739–746. https://doi.org/10.1038/372739a0.
Legler, D.F., Thelen, M., 2016. Chemokines: chemistry, biochemistry and biological function. Chimia (Aarau) 70, 856–859. https://doi.org/10.2533/chimia.2016.856.
Lendeckel, U., Venz, S., Wolke, C., 2022. Macrophages: shapes and functions. Chem texts. 8, 12. doi: 10.1007/s40828-022-00163-4.
Liu, W., Jiang, L., Bian, C., Liang, Y., Xing, R., Yishakea, M., Dong, J., 2016. Role of CX3CL1 in diseases. Arch. Immunol. Ther. Exp. (Warsz). 64, 371–383. https://doi.org/10.1007/s00005-016-0395-9.
Luo, L.J., Liu, F., Lin, Z.K., Xie, Y.F., Xu, J.L., Tong, Q.C., Shu, R., 2012. Genistein regulates the IL-1 beta induced activation of MAPKs in human periodontal ligament cells through G protein-coupled receptor 30. Arch. Biochem. Biophys. 522, 9–16. https://doi.org/10.1016/j.abb.2012.04.007.
Melby, J.O., Nard, N.J., Mitchell, D.A., 2011. Thiazole/oxazole-modified microcins: Complex natural products from ribosomal templates. Curr. Opin. Chem. Biol. 15, 369–378. https://doi.org/10.1016/j.cbpa.2011.02.027.
Mitchell, D.A., Lee, S.W., Pence, M.A., Markley, A.L., Limm, J.D., Nizet, V., Dixon, J.E., 2009. Structural and functional dissection of the heterocyclic peptide cytotoxin streptolysin S. J. Biol. Chem. 284, 13004–13012. https://doi.org/10.1074/jbc.M900802200.
Miyoshi-Akiyama, T., Takamatsu, D., Koyanagi, M., Zhao, J., Imanishi, K., Uchiyama, T., 2005. Cytocidal effect of *Streptococcus pyogenes* on mouse neutrophils *in vivo* and the critical role of streptolysin S. J. Infect. Dis. 192, 107–116. https://doi.org/10.1086/430617.
Mohammadi, M.H., Kariminik, A., 2021. CC and CXC chemokines play key roles in the development of polyomaviruses related pathological conditions. Virol. J. 18, 111. https://doi.org/10.1186/s12985-021-01582-4.
Morii, K., Fujiwara, S., Nakamura, S., Okushin, H., 2018. *Streptococcus anginosus* group-associated pyogenic liver abscess. Intern. Med. 57, 2271–2272. https://doi.org/10.2169/internalmedicine.0212-17.
Nagamune, H., Ohnishi, C., Katsuura, A., Fushitani, K., Whiley, R.A., Tsuji, A., Matsuda, Y., 1996. Intermedilysin, a novel cytotoxin specific for human cells secreted by *Streptococcus intermedius* UNS46 isolated from a human liver abscess. Infect. Immun. 64, 3093–3100. https://doi.org/10.1128/iai.64.8.3093-3100.1996.
Nizet, V., Beall, B., Bast, D.J., Datta, V., Kilburn, L., Low, D.E., De Azavedo, J.C., 2000. Genetic locus for streptolysin S production by group A streptococcus. Infect. Immun. 68, 4245–4254. https://doi.org/10.1128/IAI.68.7.4245-4254.2000.
Pilarczyk-Zurek, M., Sitkiewicz, I., Koziel, J., 2022. The clinical view on *Streptococcus anginosus* group - opportunistic pathogens coming out of hiding. Front. Microbiol. 13, 956677 https://doi.org/10.3389/fmicb.2022.956677.
Pinho-Ribeiro, F.A., Baddal, B., Haarsma, R., O'Seaghdha, M., Yang, N.J., Blake, K.J., Portley, M., Verri, W.A., Dale, J.B., Wessels, M.R., Chiu, I.M., 2018. Blocking neuronal signaling to immune cells treats streptococcal invasive infection. Cell 173, 1083–1097. https://doi.org/10.1016/j.cell.2018.04.006 e22.
Rawla, P., Vellipuram, A.R., Bandaru, S.S., Raj, J.P., 2017. Colon carcinoma presenting as *Streptococcus anginosus* bacteremia and liver abscess. Gastroenterology. Res. 10, 376–379. https://doi.org/10.14740/gr884w.
Segura, M., Vadeboncoeur, N., Gottschalk, M., 2002. CD14-dependent and -independent cytokine and chemokine production by human THP-1 monocytes stimulated by *Streptococcus suis* capsular type 2. Clin. Exp. Immunol. 127, 243–254. https://doi.org/10.1046/j.1365-2249.2002.01768.x.
Simonini, A., Moscucci, M., Muller, D.W., Bates, E.R., Pagani, F.D., Burdick, M.D., Strieter, R.M., 2000. IL-8 is an angiogenic factor in human coronary atherectomy tissue. Circulation 101, 1519–1526. https://doi.org/10.1161/01.cir.101.13.1519.
Sukeno, A., Nagamune, H., Whiley, R.A., Jafar, S.I., Aduse-Opoku, J., Ohkura, K., Maeda, T., Hirota, K., Miyake, Y., Kourai, H., 2005. Intermedilysin is essential for the invasion of hepatoma HepG2 cells by *Streptococcus intermedius*. Microbiol. Immunol. 49, 681–694. https://doi.org/10.1111/j.1348-0421.2005.tb03647.x.
Sumitomo, T., Nakata, M., Higashino, M., Jin, Y., Terao, Y., Fujinaga, Y., Kawabata, S., 2011. Streptolysin S contributes to group A streptococcal translocation across an epithelial barrier. J. Biol. Chem. 286, 2750–2761. https://doi.org/10.1074/jbc.M110.171504.
Tabata, A., Nakano, K., Ohkura, K., Tomoyasu, T., Kikuchi, K., Whiley, R.A., Nagamune, H., 2013. Novel twin streptolysin S-like peptides encoded in the *sag* operon homologue of beta-hemolytic *Streptococcus anginosus*. J. Bacteriol. 195, 1090–1099. https://doi.org/10.1128/JB.01344-12.
Tabata, A., Sato, Y., Maya, K., Nakano, K., Kikuchi, K., Whiley, R.A., Ohkura, K., Tomoyasu, T., Nagamune, H., 2014. A streptolysin S homologue is essential for β-hemolytic *Streptococcus constellatus* subsp. *constellatus* cytotoxicity. Microbiology (Reading) 160, 980–991. https://doi.org/10.1099/mic.0.075580-0.
Tabata, A., Yamada, T., Ohtani, H., Ohkura, K., Tomoyasu, T., Nagamune, H., 2019. β-Hemolytic *Streptococcus anginosus* subsp. *anginosus* causes streptolysin S-dependent cytotoxicity to human cell culture lines *in vitro*. J. Oral Microbiol. 11, 1609839 https://doi.org/10.1080/20002297.2019.1609839.
Tabata, A., Nagamune, H., 2021. Diversity of β-hemolysins produced by the human opportunistic streptococci. Microbiol. Immunol. 65, 512–529. https://doi.org/10.1111/1348-0421.12936.
Uciechowski, P., Dempke, W.C.M., 2020. Interleukin-6: A masterplayer in the cytokine network. Oncology. 98, 131–137. https://doi.org/10.1159/000505099.
Uhlén, P., Laestadius, A., Jahnukainen, T., Söderblom, T., Bäckhed, F., Celsi, G., Brismar, H., Normark, S., Aperia, A., Richter-Dahlfors, A., 2000. Alpha-haemolysin of uropathogenic *E. coli* induces Ca^{2+} oscillations in renal epithelial cells. Nature 405, 694–697. https://doi.org/10.1038/35015091.
Yamada, T., Yamamori, Y., Matsuda, N., Nagamune, H., Ohkura, K., Tomoyasu, T., Tabata, T., 2023. Streptolysin S induces pronounced calcium-ion influx-dependent expression of immediate early genes encoding transcription factors. Sci. Rep. 13, 13720. https://doi.org/10.1038/s41598-023-40981-1.
Yokohata, S., Ohkura, K., Nagamune, H., Tomoyasu, T., Tabata, A., 2023. Human serum albumin stabilizes streptolysin S activity secreted in the extracellular milieu by streptolysin S-producing streptococci. Microbiol. Immunol. 67, 58–68. https://doi.org/10.1111/1348-0421.13042.
Yoshimura, T., 2015. Discovery of IL-8/CXCL8 (The Story from frederick). Front. Immunol. 6, 278. https://doi.org/10.3389/fimmu.2015.00278.