Staphylococcal superantigen-like protein 10 induces necroptosis through TNFR1 activation of RIPK3-dependent signal pathways

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Staphylococcal aureus (S. aureus) infection can lead to a wide range of diseases such as sepsis and pneumonia. Staphylococcal superantigen-like (SSL) proteins, expressed by all known S. aureus strains, are shown to be involved in immune evasion during S. aureus infection. Here, we show that SSL10, an SSL family protein, exhibits potent cytotoxicity against human cells (HEK293T and HUVEC) by inducing necroptosis upon binding to its receptor TNFR1 on the cell membrane. After binding, two distinct signaling pathways are activated downstream of TNFR1 in a RIPK3-dependent manner, i.e., the RIPK1-RIPK3-MLKL and RIPK3-CaMKII-mitochondrial permeability transition pore (mPTP) pathways. Knockout of ssl10 in S. aureus significantly reduces cytotoxicity of the culture supernatants of S. aureus, indicating that SSL10 is involved in extracellular cytotoxicity during infection. We determined the crystal structure of SSL10 at 1.9 Å resolution and identified a positively charged surface of SSL10 responsible for TNFR1 binding and cytotoxic activity. This study thus provides the description of cytotoxicity through induction of necroptosis by the SSL10 protein, and a potential target for clinical treatment of S. aureus-associated diseases.
S. aureus (S. aureus) is a prevalent and opportunistic pathogen that causes a wide range of diseases such as sepsis, pneumonia, endocarditis, and osteomyelitis, threatening the health of both humans and animals. Moreover, S. aureus is among the most clinically challenging pathogens worldwide because of its propensity for rapid development and sharing of antibiotic resistance. Although antibiotic treatments saved millions of lives worldwide, the continued rise in antibiotic resistance has become a major public health concern. S. aureus bacteremia can range between 15 and 50%. Six to 12% of people with S. aureus bacteremia can die of the infection, and S. aureus is also responsible for many cases of sepsis. In severe sepsis caused by S. aureus, vascular permeation, immunosuppression, and organ failure are usually present, which strongly suggests the occurrence of cell death including necroptosis.

Staphylococcal superantigen like (SSL) proteins comprise a family of 14 member proteins with sequences and structures homologous to superantigens but lacking superantigenic activities. The 14 ssl genes are encoded at two different loci, with ssl1–ssl11 in the genomeic island VsaA, and ssl12–ssl14 in the immune evasion cluster. Among all the SSL proteins, SSL10 is a well-studied member that has been found in most human and animal isolates of S. aureus and involved in several pathological processes. For example, SSL10 can bind to CXCR4 and then inhibit migration of leukemia cells. SSL10 also blocks the interaction between IgG and complement component C1q that consequently prevent the activation of the classical complement pathway. Furthermore, SSL10 interacts with prothrombin and factor Xa to impair blood coagulation. It has also been shown that SSL10 could interfere with host cell inflammation via binding to ERK. All these studies suggest that SSL10 possesses multiple functions during S. aureus infection, highlighting the importance of SSL10.

Although SSL proteins have diverse functions in modulating host response to S. aureus infection, it remains unknown whether SSL family proteins can induce cytotoxicity. In the present study, we demonstrate that SSL10 exhibits potent cytotoxicity toward HEK293T and HUVEC cell growth by inducing cellular necroptosis and contributes to the cytotoxicity induced by S. aureus. Mechanistically, SSL10 induces necroptosis through binding to TNFR1 and activating two distinct signal pathways downstream RIPK3. This study provides evidence that SSL10 is a cytotoxic virulence factor that may serve as a therapeutic target in S. aureus infections.

**Results**

**SSL10 induces cell necrosis.** To determine the cytotoxicity of SSLs on host cells, we cloned and expressed all of the 14 SSL members in E. coli, SSL3, SSL7, SSL8, SSL10, and SSL11 proteins were successfully purified with high quality for cytotoxicity assays (Supplementary Methods). Human umbilical vein endothelial cells (HUVEC) were treated with these purified recombinant SSL proteins for different time periods. As determined by MTS assay (Supplementary Fig. 1), SSL10, but not SSL3, SSL7, SSL8 or SSL11, significantly reduced the cell activity of HUVEC after 2-day treatment. Furthermore, SSL10 treatment decreased cell viability in a dose- and time-dependent manner in human embryonic kidney cells (HEK293T) and HUVEC (Fig. 1a). Most non-viable cells were PI-positive but Annexin V-negative when detected by flow cytometry, suggesting that cell death induced by SSL10 is most likely to be necrosis (Fig. 1b, c and Supplementary Fig. 2).

Cell necrosis was further confirmed by transmission electron microscopy in which the cells exhibited a typical necrotic phenotype, including cytoplasmic lightening, swollen organelle, and membrane rupture (Fig. 1d). In addition, pretreatment with pan-caspase inhibitor Z-VAD-fmk had little effect on SSL10-induced LDH release, indicating that SSL10 induces necrosis but not apoptosis or pyroptosis (Fig. 1e).

Because SSL10 is a virulence factor secreted by S. aureus, we also detected the effects of SSL10 in the supernatants of S. aureus. We quantified the cytotoxicity of supernatants from wild type (WT) S. aureus strain (NCTC 8325), its ssl7 knockout, ssl10 knockout, or ssl10 complementation strain toward HEK293T and HUVEC. Compared with medium-treated cells, LDH release was significantly increased in cells treated with supernatants from the WT S. aureus. ssl10 knockout but not ssl7 knockout significantly hampered the LDH release induced by S. aureus supernatant, which can be rescued by ssl10 complementation to a level similar to WT S. aureus (Fig. 1f). All these data indicate that SSL10 from S. aureus can induce cell necrosis of both HEK293T and HUVEC.

**SSL10 induces necroptosis via the RIPK3-dependent pathway.** To explore the underlying mechanism by which SSL10 induced necrosis, we pretreated the HEK293T or HUVEC cells with a specific RIPK1 inhibitor (Nec-1s) or RIPK3 inhibitor (GSK872) prior to SSL10 exposure. As determined by LDH release, Nec-1s could partially attenuate the effects of SSL10, while GSK872 almost completely inhibited the necrotic effects of SSL10 (Fig. 2a). These data suggest that SSL10 may induce RIPK3-dependent cellular necroptosis.

To further test this hypothesis, we generated knockout cell lines for key genes involved in the necroptosis pathway via CRISPR-Cas9 in both HEK293T and HUVEC cells (Supplementary Fig. 3 and Table 1). Consistent with the effects of inhibitor treatment, knockout of RIPK3 almost completely inhibited SSL10-induced
necrosis, while knockout of RIPK1 or MLKL only resulted in partial inhibition (Fig. 2b–d). In addition, transient complementation with RIPK3 in Ripk3−/− HEK293T cells led to robust necrosis, evidenced by the release of LDH (Fig. 2e). Together, these data indicate that SSL10 could induce RIPK3-dependent necroptosis in both HEK293T and HUVEC.

CaMKII activation and mPTP opening also contribute to SSL10-induced necroptosis. Previous studies have reported that RIPK1 can form a complex with RIPK3, which further activates MLKL, resulting in necroptosis of several types of cells. However, in the present study, we found that inhibition or knockout of RIPK1 or MLKL could not completely inhibit SSL10-
induced necroptosis, suggesting that SSL10-induced necroptosis may also depend on other RIPK3-mediated pathways independent of RIPK1 and MLKL.

In addition to MLKL, RIPK3 has been reported to phosphorylate CaMKII to induce the opening of mPTP channels, for example, leading to necroptosis in cardiomyocytes. To explore whether CaMKII was involved in SSL10-induced necroptosis, HEK293T and HUVEC cells were treated with KN-93, a selective inhibitor of CaMKII prior to SSL10 treatment. As assessed by the release of LDH and ATP, inhibition of CaMKII profoundly abrogated SSL10-induced necroptosis (Fig. 3a, b). Phospho-CaMKII levels were also significantly increased after SSL10 treatment (Fig. 3c), which suggested the involvement of CaMKII in SSL10-induced necroptosis.

To further identify the downstream effector of CaMKII, we pretreated HEK293T and HUVEC cells with CsA, an inhibitor of mPTP opening, and found that CsA efficiently blocked SSL10-induced LDH release (Fig. 3d). In addition, SSL10 treatment led to mitochondrial depolarization, which was significantly hampered in the absence of RIPK3, evidenced by the recovery in mitochondrial membrane potential ($\Delta \Psi_m$) (Fig. 3e). These data indicate that CaMKII-mPTP is likely to be a primary candidate downstream of RIPK3 in SSL10-induced necroptosis.

### SSL10 induces necroptosis by direct interaction with the TNFR1 extracellular domain (TNFR1EC).

Necroptosis is initiated through ligand binding to several receptors including TNFR1, toll-like receptors (TLR3 and TLR4), and interferon receptors. To explore whether SSL10 induces necroptosis by interacting with membrane receptors, SSL10 localization was examined by real-time live-cell analysis and scanning confocal microscopy. Notably, GFP-tagged SSL10 was found to be enriched on the HUVEC cell membrane within the first 30 min of treatment, suggesting that SSL10 may bind to a cell surface receptor (Supplementary Fig. 4). Similarly, His-tagged SSL10 proteins, but not SSL7 proteins, were found on the HEK293T cell surface as detected by flow cytometry (Fig. 4a).

To further determine which membrane receptor was involved in SSL10-induced necroptosis, inhibitors against TLR4 (TAK-242) and interferon receptor IFNAR1 (IFN alpha-IFNAR-IN-1) were used to pre-treat HEK293T cells before SSL10 exposure. However, SSL10-induced necroptosis was not affected by these inhibitors, indicating that SSL10-induced necroptosis is independent of TLR4 or IFNAR1 (Supplementary Fig. 5). In addition to these two receptors, TNFR1 is a well characterized receptor that can trigger necroptosis through activating RIPK3. To test whether TNFR1 participated in SSL10-induced necroptosis, we knocked out TNFR1 in HEK293T and HUVEC cells via CRISPR-Cas9 and found that depletion of TNFR1 significantly decreased the binding of SSL10 to the cells (Fig. 4a), and blocked SSL10-induced necroptosis, as indicated by the significantly decreased release of LDH (Fig. 4b).

Overall structure of SSL10. To further understand the molecular mechanisms driving the SSL10 activation of necroptosis via binding to TNFR1, we next determined the crystal structure of SSL10 by molecular replacement at 1.9 Å resolution. X-ray diffraction data and structure refinement statistics are shown in Table 2. SSL10 existed as a monomer in both solution and crystal structure (Supplementary Fig. 7). Two SSL10 molecules were observed in one asymmetric unit adopting approximately identical structures, with the RMSD value of 0.254 Å when the two molecules were aligned (Supplementary Fig. 8a). In light of these results, we selected molecule B for further investigation. SSL10 exhibited a typical superantigen-like structure, consisting of two distinct domains separated by a flexible linker region. The N-terminal OB-fold domain (residues 43–123) contains one $\alpha$-helix, eight $\beta$-strands, and one $\beta$10 helix, while the C-terminal $\beta$-grasp domain (residues 133–227) consists of one $\alpha$-helix, seven $\beta$-strands, and two $\beta_10$ helices (Fig. 5a). Structural comparison of SSL10 with other SSL proteins (i.e., SSL3, SSL4, SSL5, SSL7, SSL8, and SSL11) revealed that the SSL proteins share similar folds (Supplementary Fig. 8b) but are quite different in electrostatic surface potential (Fig. 5b) and Supplementary Fig. 8c), which might be responsible for the diverse binding partners and functions of the SSL proteins.

Both the N- and C-terminal domains of SSL10 contribute to its cytotoxicity. To investigate which domain or domains of SSL10 may be critical for its cytotoxicity, we generated variants of SSL10 with the N- and C-terminal domains swapped between SSL7. We designated the two newly generated chimeric proteins as...

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**Fig. 1 SSL10 induces necrosis in HEK293T and HUVEC cells.**

- **a** HEK293T and HUVEC cells were treated with different concentrations of SSL10 for different time periods, as indicated. The cell viability was determined by MTS assay. HEK293T (**b**) and HUVEC (**c**) cells were collected after treatment with 2 μM SSL10 for 48 h at 37 °C and 5% CO2, and then detected by flow cytometry using Annexin V/PI staining. The dot plot (left) is representative of three independent experiments, and the quantitative results are shown as a bar graph (right). **d** Transmission electron microscopy images of HEK293T and HUVEC cells treated with or without 2 μM SSL10 for 48 h at 37 °C and 5% CO2. Red letters with arrows indicate characteristic features of necrotic morphology: **a** cytoplasmic lightening; **b** swollen organelle; **c** membrane rupture. **e** Cells were pretreated with 10 μM Z-VAD-fmk for 30 min at 37 °C and 5% CO2, and then stimulated with 2 μM SSL10. The release of LDH was detected. **f** The supernatant of wild type *S. aureus* NCTC 8225, SSL10 knockout, ssl7 knockout or ssl10 complementation bacteria was used to treat HEK293T and HUVEC cells for 48 h at 1:10 dilution at 37 °C and 5% CO2. LDH released from the cells was evaluated. WT wild type. Cells treated with 2 μM SSL7 were used as a negative protein control throughout the experiments. All data represent the means ± SD calculated from three independent experiments. *p* < 0.05 compared to the WT or SSL10 knockout. **g** *S. aureus* supernatant treated group as indicated. n.s. not significant, by one-way ANOVA (**b–f**) or two-way ANOVA (**a**).
Fig. 2 SSL10 triggers RIPK3-dependent necroptosis of HEK293T and HUVEC cells. a HEK293T and HUVEC cells were pretreated with 50 μM Nec-1s or 50 μM GSK'872 for 30 min and then stimulated with 2 μM SSL10 for 48 h at 37 °C and 5% CO2. The release of LDH was detected. b Ripk1, Ripk3, or Mlkl knockout (KO) HEK293T cells were treated with 2 μM SSL10 for 48 h at 37 °C and 5% CO2, and the LDH release was detected. c Wild type (WT) or Ripk3 KO HUVEC were treated with 2 μM SSL10 for 48 h at 37 °C and 5% CO2, and the LDH release was detected. d WT or Ripk3 KO HEK293T cells were treated with SSL10 for 48 h and the cell viability was measured by flow cytometry after Annexin V/PI staining. The dot plot (left) is representative of three independent experiments, and the results of quantification (right) are shown as a bar graph. e WT or Ripk3 KO HEK293T cells with or without transient complementation of Ripk3 were treated with 2 μM SSL10 for 48 h at 37 °C and 5% CO2, and the LDH release was detected. Cells treated with 2 μM SSL7 were used as a negative protein control throughout the experiments. All data are presented as the means ± SD calculated from three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 compared to the Ctrl cells (buffer-treated cells). #p < 0.05; ##p < 0.01; ###p < 0.001 compared to the SSL10 treated group, by one-way (a and d) or two-way ANOVA (b, c, and e).

Table 1 Sequences of sgRNA for CRISPR-Cas9 genome editing.

| Genes    | sgRNA         | Oligo 1 (5'-3')                             | Oligo 2 (5'-3')                             |
|----------|---------------|---------------------------------------------|---------------------------------------------|
| Ripk1    | sgRNA-1       | 5'-GACGTGAAGAGTTAAAAGGT-3'                  | 5'-ACCTTTAAACTCTTTCAGTCT-3'                 |
|          | sgRNA-2       | 5'-AGTACTCCCGCTTTCTGAAA-3'                 | 5'-TTTACAGAAACGAGGTACT-3'                  |
| Ripk3    | sgRNA-1       | 5'-TTCAGACCGCGCAAAGGAGG-3'                 | 5'-CTCCTTTGGCGCTGCTGAA-3'                  |
|          | sgRNA-2       | 5'-GGACCCAGAGGCTGGAGTC-3'                  | 5'-TGACGTGACGCTGGTGGTCC-3'                 |
| Mlkl     | sgRNA-1       | 5'-GCTGATCACGCGAGGAGG-3'                   | 5'-GTCCTCCTGCTGCTGACG-3'                   |
|          | sgRNA-2       | 5'-GCTGATCACGCGAGGAGG-3'                   | 5'-GTCCTCCTGCTGCTGACG-3'                   |
| Tnfrsf1a | sgRNA-1       | 5'-GGTGGGAATATACCCCTTAC-3'                 | 5'-CTGAGGGGATATATCCCGACC-3'                |
|          | sgRNA-2       | 5'-GGTGGCACCCGCTATCCAGG-3'                 | 5'-CTGATAGGGGATGCTGCCACC-3'                |
SSL7 × 10 and SSL10 × 7, with SSL7 × 10 containing the SSL7 N-terminus and the SSL10 C-terminus, and vice versa (Fig. 5c). We found that both of the chimeric proteins induced a marked release of LDH, but were significantly less potent than SSL10 (Fig. 5d). In agreement with these results, both of the chimeric proteins could still bind to TNFR1ECD with weaker binding activity compared to SSL10 (Fig. 5e). These data indicate that both the N- and C-terminal domains of SSL10 interact with TNFR1ECD and participate in SSL10-induced necroptosis.

Predicted binding interface between SSL10 and TNFR1. To analyze the regions in TNFR1ECD mediating its interaction with SSL10, we constructed 4 MBP-tagged TNFR1 deletion mutants (i.e., ΔCRD1, ΔCRD2, ΔCRD3, and ΔCRD4) according to its four extracellular CRDs33. We found that ΔCRD2 mutant of TNFR1ECD, but not the other three mutants, showed significantly decrease in its binding ability to SSL10 (Fig. 6a). Such decrease in the interaction between ΔCRD2 mutant of TNFR1ECD and SSL10 was further confirmed by the decreased binding affinity (with KD value of 30.3 ± 3.55 μM) as determined by ITC (Fig. 6b). Consistently, compared to WT TNFR1ECD, the inhibitory effects to SSL10 induced-cytotoxicity of ΔCRD2 mutant was also significantly decreased (Fig. 6c). These results confirm that the second CRD region of TNFR1ECD is important for its interaction with SSL10.

To further understand the molecular mechanism underlying the interaction between SSL10 and TNFR1, the structure of TNFR1ECD (PDB code: 1EXT) was docked onto the structure of SSL10 using the HawkDock webserver (http://cadd.zju.edu.cn/hawkdock/)34.
Fig. 4 SSL10 induces necroptosis by direct interacting with the extracellular domain of TNFR1 (TNFR1ECD). a Wild type (WT) or Tnfrsf1a knockout (KO) HEK293T cells were treated with SSL10 at different concentrations for 30 min, and the SSL proteins bound to the cell surface were detected by flow cytometry after FITC-conjugated anti-His-tag antibody staining. The dot plot (left) is representative of three independent experiments, and the results of quantification (right) are shown as a bar graph.

b Depolarization of the mitochondrial membrane of WT or Tnfrsf1a KO HEK293T cells treated with SSL10 was measured by flow cytometry after JC-1 staining. The dot plot (left) is representative of three independent experiments, and the results of quantification (right) are shown as a bar graph.

c WT or Tnfrsf1a KO HEK293T cells were challenged with 2 µM SSL10 for 48 h and the cell viability was measured by flow cytometry after Annexin V/PI staining. The dot plot (left) is representative of three independent experiments, and the results of quantification (right) are shown as a bar graph.

d KO HEK293T cells were treated with SSL10 at different concentrations for 30 min, and the SSL proteins bound to the cell surface were detected by flow cytometry after FITC-conjugated anti-His-tag antibody staining. The dot plot (left) is representative of three independent experiments, and the results of quantification (right) are shown as a bar graph.

e Immunoblotting of SSL10 after pull-down with MBP-TNFR1ECD. f ICT assays for SSL10 binding to MBP-TNFR1ECD or MBP control protein. Cells treated with 2 µM SSL7 were used as a negative protein control throughout the experiments. All data are presented as the means ± SD from three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 compared to the Ctrl cells (buffer-treated cells). *p < 0.05; **p < 0.01; ***p < 0.001 compared to the SSL10 treated group, by two-way ANOVA.
The binding interfaces of the top 10 predicted models were further analyzed by Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) method\(^1\). The binding free energies of these top 10 models ranged from \(-37.63\) kcal/mol to \(-7.51\) kcal/mol (Supplementary Table 1). As shown previously, both the N- and C-terminal domains of SSL10 contribute to TNFR1ECD interaction and necroptosis induction (Fig. 5), whereas the recognition of SSL10 is largely mediated by CRD2 of TNFR1ECD (Fig. 6a–c). Therefore, the models with the interface formed by both the N- and C-terminal domain of SSL10 and the CRD2 region of TNFR1ECD, which were model 1, 2, 4, and 8, were included for further analysis (Supplementary Fig. 9a). In each model, TNFR1ECD binds to a positively charged region on SSL10, suggesting the recognition of TNFR1 by SSL10 is dominantly mediated by electrostatic interactions (Fig. 6d, e and Supplementary Fig. 9b).

To assess the reliability of these models, four SSL10 mutants, named M1, M2, M4, and M8 were generated by replacing the important residues buried in the interface by amino acid residues either with the opposite charge or the corresponding counterparts present in SSL3, SSL7, SSL8, or SSL11 (Supplementary Table 2, Figs. 6d, e, S9b and S10a). Indicated by size exclusion chromatography, we obtained aggregated M1, M2, M4 and M8 proteins which were improperly folded (Supplementary Fig. 10b). Therefore, we used the monomeric M1 and M2 for MBP pull-down assays. As shown in Fig. 6f, the binding of M1 to TNFR1ECD was abolished, while M2 showed comparable binding ability to TNFR1ECD as WT SSL10. Disruption of the interaction between M1 and TNFR1ECD was further confirmed by the ITC assay (Fig. 6g). Consistently, the release of LDH from HEK293T cells treated by M1 was significantly decreased compared to WT SSL10 (Fig. 6h). These results suggested a binding surface on SSL10 responsible for TNFR1 interaction and necroptosis induction.

### Discussion

Previous studies on SSL10 suggested that SSL10 contributed to S. aureus pathogenicity by targeting different cellular responses, including the classical complement activation pathway, the migration of T cells, the interaction between complement C1q and IgG, and the Fc-receptor-mediated phagocytosis of neutrophils\(^24\)–\(^27\). In addition, recent publication showed that SSL10 binds to ERK2 to interfere with host cell inflammation\(^28\). Here, our data demonstrated the cytotoxicity of SSL10 by triggering necroptosis via activation of two distinct signaling pathways upon binding to TNFR1 in HEK293T and HUVEC cells.

SSL10, like other SSL protein family members, adopts conserved structure with an N-terminal OB-fold domain and a C-terminal β-grasp domain\(^36\). SSL10 has been shown to interact with various partners on distinct binding sites to perform different functions. For example, SSL10 binds to IgG1 predominantly via the N-terminal OB-fold domain to inhibit the FcR-mediated phagocytosis\(^24\), while both OB-fold and β-grasp domains contribute to the interaction of SSL10 with the γ-carboxyglutamic acid (Gla) domain of prothrombin\(^27\). In this study, we identified a TNFR1 binding site on SSL10 surface involving both OB-fold and β-grasp domains based on the docking model of SSL10-TNFR1ECD complex, as well as the mutagenesis and cytotoxic analysis (Fig. 6d–h). Interestingly, the binding site for TNFR1ECD of SSL10 overlap with the binding site (amino acid residues 54-81 and 195-227) to prothrombin\(^37\). LDH assays reveal that the Gla domain of prothrombin inhibits the cytotoxicity of SSL10 in a dose-dependent manner (Supplementary Fig. 6c). Therefore, under certain circumstances, prothrombin might compete with TNFR1 for interaction with SSL10.

Damage of endothelial cells can facilitate the spread of S. aureus into the bloodstream, leading to the development of septic shock and organ failure\(^38\),\(^39\). Virulence factors from S. aureus play important roles in the development and progression of sepsis through multiple mechanisms including disrupting various types of host cells\(^1\),\(^6\),\(^40\)–\(^43\). Reijer et al. characterized the serial levels of IgG and IgA antibodies against 56 staphylococcal antigens in multiple serum samples of 21 patients with a S. aureus bacteremia. Their data showed that an increase in IgG levels against SSL10 was observed at some time point after the onset of bacteremia in 95 to 100% of all patients\(^44\). Hemostatic abnormalities frequently occur during sepsis and are most often attributed to disseminated intravascular coagulation. Draaijers et al. reported the case of a patient with severe coagulopathy acquired during fulminant S. aureus sepsis and speculated that inhibition of coagulation factor X by S. aureus SSL10 is the most likely cause of the acquired coagulopathy in their patient\(^1\). In addition to the previously identified function, our study revealed a new function of SSL10 to trigger necroptosis of endothelial and epithelial cells in a RIPK3-dependent manner. Although RIPK3 is expressed at an extremely low level in endothelial and epithelial cells, including HUVEC, and HEK293T, its importance in vivo cannot be excluded\(^45\),\(^47\). Genetic evidence showed that RIPK3 deficiency leads to reduced endothelial cell permeability or necroptosis, thereby suppressing tumor metastasis\(^38\),\(^49\). Furthermore, RIPK3 can be induced or upregulated under certain conditions, which confers cells sensitive to RIPK3-dependent necroptosis\(^48\). Notably, we found an SSL10-induced increase in the protein level of RIPK3 in HEK293T and HUVEC cells (Supplementary Fig. 11), indicating the important role of RIPK3 on SSL10-induced necroptosis.

### Table 2 Crystallographic data collection and structure refinement.

| PDB code  | SSL10       |
|-----------|-------------|
| Data collection | 6LWT\(^a\)     |
| Space group  | P2\(_1\) 2\(_1\) 2\(_1\) |
| Cell dimensions |
| a, b, c (Å) | 41.24, 71.21, 140.66 |
| α, β, γ (°) | 90, 90, 90 |
| Resolution (Å) | 50.0-1.90 (1.93-1.90)\(^b\) |
| Wavelength (Å) | 0.9778 |
| R<sub>sym</sub> or R<sub>merge</sub> (%) | 9.0 (29.4) |
| Overall I/σ(I) | 16.2 (7.0) |
| Completeness (%) | 100.0 (100.0) |
| Redundancy | 6.4 (6.6) |
| Refinement |
| Resolution (Å) | 39.58-1.90 |
| No. reflections | 31,821 |
| R<sub>work</sub>/R<sub>free</sub> (%) | 20.94/24.62 |
| No. atoms |
| Protein/Water | 3127/57 |
| Average B-factors (Å\(^2\)) | 30.858/27.255 |
| R.m.s. deviations |
| Bond lengths (Å) | 0.0088 |
| Bond angles (°) | 1.3524 |
| Ramachandran plot\(^c\) |
| Most favored regions (%) | 96.23 |
| Allowed regions (%) | 3.77 |
| Generously allowed regions (%) | 0 |

\(^a\)One single crystal of SSL10 was used for the structure determination.

\(^b\)The values in parentheses refer to the highest resolution shell.

\(^c\)Statistics for the Ramachandran plot from an analysis using MolProbity.
addition, we also observed that mRNA levels of ssl10 in the strains isolated from patients with hypoproteinaemia, septic shock or multiple organ dysfunction syndrome (MODS) were significantly higher than that in strains from patients without these diagnoses, whereas the mRNA levels of ssl7 in these strains were similar (Supplementary Methods, Supplementary Fig. 12). Since hypoproteinaemia, septic shock or MODS are the hallmarks of endothelial damage, it is quite possible that SSL10 may be involved in the progression of S. aureus-induced bacteremia via different mechanisms, including triggering necroptosis of endothelial cells.

In summary, our study demonstrates that SSL10 triggers a signal cascade leading to necroptosis via its direct interaction with TNFR1. Moreover, this signal cascade is activated in a RIPK3-dependent manner and transduced through two independent signaling pathways (Fig. 7). We also identified a binding interface involving a positively charged surface of SSL10 and the second extracellular cysteine-rich domain of TNFR1. This study, in combination with other previous reports, provides strong evidence that SSL10 contributes to S. aureus infection via multiple mechanisms. This study also suggests that SSL10 may serve as a potential reliable biomarker and therapeutic target in S. aureus-associated infection and diseases.

**Methods**

**Reagents and cell culture.** HUVEC line and human embryonic kidney cell line (HEK293T) were obtained from Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum and incubated at 37°C in humidified incubator with 5% CO2.

*S. aureus* RN4220 and RN4220 were kindly provided by Dr. Min Li (Renji Hospital, Shanghai Jiao Tong University School of Medicine).

Primary antibodies against RIPK1 (1:1000), RIPK3 (1:1000), MLKL (1:1000), TNFR1 (1:1000), CaMKII (1:1000), phospho-CaMKII (1:1000), and GAPDH (1:1000) were purchased from Cell Signaling Technology (Danvers, MA, USA). Primary antibodies against actin and tubulin were purchased from TransGen Biotechnology (Beijing, China). Primary antibodies against His-tag (1:2000) and MBP (1:1000) were purchased from Affinity Biosciences (China) and Santa Cruz Biotechnology (Dallas, USA), respectively. The secondary antibodies including anti-rabbit IgG H&L (HRP-conjugated) and anti-mouse IgG H&L (HRP-conjugated) were obtained from Beyotime Biotechnology (Shanghai, China).

CellTiter 96® AQueous One Solution Cell Proliferation Assay and CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI) were used for MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] and lactate dehydrogenase (LDH) assays, respectively. To measure the ATP concentration in the cells, CellTiter-Glo® Luminescent Viability Assay (Promega, Madison, WI) was used, while FITC Annexin V Apoptosis Detection Kit I (BD, New Jersey, US) was used to determine the cell death by flow cytometry.

Inhibitors used in the present study are as follows: necrostatin-1 stable (Nec-1s, Biovision), GSK872 (Calbiochem), Z-VAD-fmk (Selleckchem), KN-93 (APEXBio), and cyclosporine A (CsA, APEXBio).

**Cytotoxicity assays.** HUVEC or HEK293T cells were seeded in 96-well plate (100 µl per well) at a density of 1 × 10³ or 1 × 10⁴ cells/well, 1 day prior to treatment with or without SSL10 resuspended in Opti-MEM reduced serum medium (Thermo Fisher) for 48 h. To detect the effects of inhibitors, the cells were pretreated with various inhibitors 0.5 h before 2 µM SSL10 treatment.

For MTS assay, 20 µl of CellTiter 96® AQueous One Solution reagent was added to each well and incubated for two more hours, and the optical density was measured at 490 nm with a BioTek Synergy/2 microplate reader (BioTek, Winooski, VT).

LDH release was measured according to the manufacturer’s manual. Briefly, 50 µl culture media from various treated cells was transferred to a new 96-well flat clear bottom plate, and 50 µl of the CytoTox 96® reagent was added to each sample aliquot and incubated in dark for 30 min at room temperature. To determine the maximum
LDH release, 10 μl of 10 × lysis solution was added to 100 μl cell control for 45 min before adding CytoTox 96® reagent. Finally, 50 μl of stop solution was added to each well and the absorbance at 490 nm was recorded with BioTek Synergy/2. LDH leakage was calculated as a percentage of the maximum LDH release of control (buffer-treated cells) group after subtracting the background absorbance.

To determine the ATP concentration in the cells, CellTiter-Glo® reagent was added to each well and the plate was incubated for 10 min to stabilize luminescent signal before luminescence was recorded with BioTek Synergy/2. Luminescent signals from blank wells and buffer-treated cells were used as background and maximal luminescence.

Cell death detected by flow cytometry was performed as previously described. Briefly, 48 h after treatment with SSL10, the cells were collected and washed twice with ice-cold PBS. The cells were then incubated with 5 μl FITC annexin V and 5 μl PI in 500 μl prepared assay buffer in dark for 10 min at room temperature, and
**Fig. 6 Identification of a potential binding interface between SSL10 and TNFR1.** a Immunoblotting of SSL10 after pull-down with MBP-TNFR1ECD or its four deletion mutants (i.e., ΔCRD1-4). The relative band intensities of SSL10 proteins pulled down by WT or mutant MBP-TNFR1ECD are quantitated by densitometry after normalization to their input, and then expressed as the fold of WT SSL10 pulled down by WT MBP-TNFR1ECD. b ITC assays of SSL10 binding to the TNFR1ECD mutant ΔCRD1 or ΔCRD2. c LDH released from HUVEC cells treated with 2 μM SSL10 alone or combined with 10 μM MBP-TNFR1ECD, ΔCRD1 or ΔCRD2, as indicated. d Model 1 of SSL10/TNFR1ECD complex was generated by HawkDock program with the crystal structures of SSL10 and TNFR1ECD (PDB code: 1EXT). The enlarged view shows the residues of SSL10 predicted to interact with TNFR1ECD. SSL10 and TNFR1ECD are colored cyan and light brown, respectively. e The electrostatic surface view of predicted binding site of SSL10 for TNFR1ECD is shown. The positive and negative charge are colored blue and red, respectively. f Immunoblotting of SSL10 and its mutants (M1 and M2) after pull-down with MBP-TNFR1ECD. g ITC assay of mutant M1 binding to MBP-TNFR1ECD. h LDH released from HEK293T cells treated with 2 μM SSL10 or mutant M1. All data represent the means ± SD calculated from three independent experiments. *p < 0.05; **p < 0.01, ***p < 0.001 compared to the Ctrl cells (buffer-treated cells) or as indicated. n.s. not significant, by one-way ANOVA.

**Fig. 7 Schematic diagram of the SSL10 signal cascade and subsequent cellular effects.** SSL10 secreted by S. aureus directly interacts with TNFR1 on the host cell surface and induces cell necroptosis via two distinct pathways, including the RIPK1-RIPK3-MLKL and RIPK3-CaMKII-mPTP pathways.

Neglected text: applied for flow cytometry analysis. The specific gating strategies are listed in Supplementary Fig. 13.

**Knockout and rescue of ssl7 and ssl10 in S. aureus NCTC 8325.** To explore the effect of SSL10 on the cytotoxicity of S. aureus, ssl10 or ssl7 knockout strain was constructed using the vector pKOR1. Briefly, ~1000 bp fragment upstream and downstream, respectively, of ssl10 or ssl7 was cloned to pKOR1 via lambda recombination (BP clonase enzyme mix, Invitrogen). The resulting plasmid was transferred via electroporation first to S. aureus RN4220 to modify DNA, and subsequently to S. aureus 8325. For homologous recombination and inactivation of pKOR1 into the bacterial chromosome, S. aureus NCTC 8325 was grown at 43 °C on tryptic soy agar (TSACm10), a non-permissive condition for pKOR1 replication. The cells were lysed in RIPA buffer containing proteinase inhibitor cocktail (Roche, Basel, Switzerland) when necessary. A total of 30 μg of protein (determined by BCA protein quantification kit) for each sample was separated on 10% SDS-PAGE, transferred to a PVDF membrane, immunoblotted with appropriate antibodies as indicated. Antibody binding was detected using a luminescent chemiluminescence substrates.

Transmission electron microscopy. HEK293T or HUVEC cells with or without SSL10 treatment were prefixed in Karnovsky’s solution (1% paraformaldehyde, 2% glutaraldehyde, 2 mM calcium chloride, 0.1 M cacodylate buffer, pH 7.4) for 2 h and washed with cacodylate buffer. Postfixing was carried out in 1% osmium tetroxide and 1.5% potassium ferrocyanide for 1 h. After dehydration with 50–100% alcohol, the cells were embedded in PolyBed 812 resin (Pelco, Redding, CA, USA), polymerized and then observed under a HITACHI model H-7650 electron microscope (HITACHI, Tokyo, Japan).

**Immunoblotting.** The cells were lysed in RIPK3 buffer containing phosphatase inhibitor (Sigma-Aldrich, St. Louis, MO, USA) and/or PhosSTOP Phosphatase Inhibitor (Roche, Basel, Switzerland) when necessary. A total of 30 μg of protein (determined by BCA protein quantification kit) for each sample was separated on 10% SDS-PAGE, transferred to a PVDF membrane, immunoblotted with appropriate antibodies as indicated. Antibody binding was detected using a luminescent image analyzer ImageQuant™ LAS 4000 mini (GE Healthcare Bio-Science AB, Uppsala, Sweden) after adding peroxidase-conjugated secondary antibodies and chemiluminescence substrates.

**CRISPR-Cas9 genome editing and rescue.** The E-CRISP online tool (http://www.e-crisp.org/E-CRISP/) was used to design specific single-guide RNAs targeting different genes. The 20-nucleotide guide sequence was annealed to the complementary oligos and then cloned into the pSpCas9 (BB)–2A–green fluorescent protein (GFP) plasmid (PX458; Addgene, Cambridge, MA, USA). HEK293T or HUVEC cells were transiently transfected with the CRISPR/Cas9 plasmids using Lipofectamine 3000 (Life Technologies, MA, USA) following the manufacturer’s instructions. GFP-positive single clones were sorted using SmartSampler Analyzer (Beckman, California, USA) 48 h after transfection and cultured in a 96-well plate for about 10 days before confirming the gene knockout by sequencing and immunoblotting or flow cytometry. Sequences of the single-guide RNAs for various genes are listed in Table 1. For RIPK3 rescue, RIPK3 cDNA was cloned into the pCDH-CMV-MCS-EF1-copGFP-T2A-Puro vector (Addgene, Cambridge, MA, USA). HEK293T WT and RIPK3 knockout cells were transiently transfected with the RIPK3-expression vector.
plasmids using Lipofectamine 3000, and 48 h after transfection, SSL10-induced necroptosis was detected by LDH release assay.

Mitochondrial membrane potential (ΔΨm) assay. The mitochondrial potential, which reflects mitochondrial depolarization, was detected using the mitochondrial membrane potential assay kit following the manufacturer’s instructions (Reyotome, Shanghai, China). JC-1 is a marker of mitochondrial activity. When the mitochondrial membrane potential (ΔΨm) is high, JC-1 is present as monomer and emits green fluorescence. The value of the JC-1 monomers to aggregates positive cells ratio quantifies the mitochondrial membrane depolarization. Briefly, JC-1 working solution was incubated with the cells in dark for 20 min at 37 °C. After three washes with prepared buffer, red and green fluorescence were detected on a flow cytometer using PI and FITC channels, respectively. The specific gating strategies are listed in Supplementary Fig. 13.

Protein expression and purification. DNA fragments encoding amino acid residues 31–227 of SSL10, 51–231 of SSL7, and SSL10 mutants were amplified by PCR from S. aureus strain Mu30 and cloned into the PET-22b (+) vector (Novagen) with a C-terminal 6 × His-tag. WT and mutant SSL10 and SSL7 were expressed in Escherichia coli BL21 (DE3) and induced with 0.4 mM IPTG (iso-propyl β-D-thiogalactopyranoside) for 4 h at 37 °C when OD600 reached 0.6. The cells were harvested by centrifugation at 6000 rpm for 8 min, and lysed in a French press in lysis buffer [50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 5% (v/v) Glyceral, 5 mM imidazole, 1 mM PMSF]. The lysate was centrifuged at 15,000 rpm for 30 min, and the supernatant was incubated with Ni-NTA resin for 30 min. The resin with target proteins were washed with 50 column volumes of washing buffer [50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 5% (v/v) Glyceral, 40 mM imidazole] to remove contaminants and the target protein was eluted by elution buffer [50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 5% (v/v) Glyceral, 300 mM imidazole]. The eluted protein was concentrated and further purified by Superdex 75 10/300 size exclusion column (GE Healthcare) equilibrated with the buffer containing 20 mM Tris-HCl, pH 7.5 and 200 mM NaCl. The entire protein purification procedure was carried out at 4 °C. Purity of the target protein was verified by SDS-PAGE and protein aliquots were stored at −80 °C for further use.

The DNA fragment of human TNFR1 extracellular domain (amino acid residues 22-211) was amplified by PCR using cDNA library of human spinal cord as template and cloned into the PET-28a (+) vector with an N-terminal MBP-tag, which was verified by Sanger sequencing. The recombiant protein MBP–TNFR1ΔΔCD and its CRD deletion mutants were expressed in E. coli Rosetta(II) strain and induced with 0.4 μM IPTG for 20 h at 16 °C when OD600 reached 0.6, and then were purified by MBP-affinity chromatography.

Crystallization. SSL10 was concentrated to 4.3 mg/mL (in buffer containing 20 mM Tris-HCl, pH 7.5 and 200 mM NaCl), and used for initial crystallization trials by the sitting-drop vapor-diffusion method at 16 °C with index crystallization screen kit. Crystals were obtained from the buffer containing 2.1 M DL-malic acid, pH 7.0.

Data collection, structure determination and refinement. The crystals of SSL10 were soaked in cryoprotectant buffer consisting 2.1 M DL-Malic acid, pH 7.0 and 20% Glyceral for several seconds and flash-cooled in liquid nitrogen. X-ray diffraction data was collected on beamline BL18U1 of Shanghai Synchrotron Radiation Facility. Diffraction data were processed, integrated, and scaled using HKL2000. The crystal structure of SSL10 was determined by molecular replacement using the program Phaser in the CCP4i suite with Exotoxin SACOL0473 (PDB code 6LWT). The crystal structure of SSL10 generated in this study have been deposited to the Protein Data Bank (PDB, https://www.rcsb.org) under the following accession number: PDB 1EXT. All other data are available from the corresponding authors on the reasonable request.

Data availability. The structure data of SSL10 generated in this study have been deposited to the Protein Data Bank (PDB, https://www.rcsb.org) under the following accession number: PDB code 1EXT. All raw data underlying the graphs and charts presented in the main and Supplementary Figures are present in Supplementary Data 1. Unedited gel images are included in Supplementary Fig. 14. All other data are available from the corresponding authors on the reasonable request.

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Additional information

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