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RESEARCH ARTICLE

Inhibition of Fibrinolysis by Streptococcal Phage Lysin$_{SM1}$

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ABSTRACT Expression of bacteriophage lysin$_{SM1}$ by *Streptococcus oralis* strain SF100 is thought to be important for the pathogenesis of infective endocarditis, due to its ability to mediate bacterial binding to fibrinogen. To better define the lysin$_{SM1}$ binding site on fibrinogen Aα, and to investigate the impact of binding on fibrinolysis, we examined the interaction of lysin$_{SM1}$ with a series of recombinant fibrinogen Aα variants. These studies revealed that lysin$_{SM1}$ binds the C-terminal region of fibrinogen Aα spanned by amino acid residues 534 to 610, with an affinity of equilibrium dissociation constant (K_D) of 3.23×10^{-5} M. This binding site overlaps the known binding site for plasminogen, an inactive precursor of plasmin, which is a key protease responsible for degrading fibrin polymers. When tested *in vitro*, lysin$_{SM1}$ competitively inhibited plasminogen binding to the αC region of fibrinogen Aα. It also inhibited plasminogen-mediated fibrinolysis, as measured by thromboelastography (TEG). These results indicate that lysin$_{SM1}$ is a bi-functional virulence factor for streptococci, serving as both an adhesin and a plasminogen inhibitor. Thus, lysin$_{SM1}$ may facilitate the attachment of bacteria to fibrinogen on the surface of damaged cardiac valves and may also inhibit plasminogen-mediated lysis of infected thrombi (vegetations) on valve surfaces.

IMPORTANCE The interaction of streptococci with human fibrinogen and platelets on damaged endocardium is a central event in the pathogenesis of infective endocarditis. *Streptococcus oralis* can bind platelets via the interaction of bacteriophage lysin$_{SM1}$ with fibrinogen on the platelet surface, and this process has been associated with increased virulence in an animal model of endocarditis. We now report that lysin$_{SM1}$ binds to the αC region of the human fibrinogen Aα chain. This interaction blocks plasminogen binding to fibrinogen and inhibits fibrinolysis. *In vivo*, this inhibition could prevent the lysis of infected vegetations, thereby promoting bacterial persistence and virulence.

KEYWORDS *Streptococcus mitis*, fibrinogen, fibrinolysis, infective endocarditis, plasminogen, thromboelastography

Infective endocarditis (IE) is a life-threatening disease of cardiac valves that can lead to complications such as congestive heart failure and stroke and has an overall mortality rate of 30% (1, 2). The pathogenesis of IE is a complex process, involving numerous host-pathogen interactions (1, 3). A key interaction for disease establishment and progression is the binding of microbes to human components, including platelets, fibrinogen, fibrin, and fibronectin on damaged endocardium (3–11).

Streptococcus oralis and Streptococcus mitis are closely related members of the oral microbiome. These two species cannot be reliably distinguished by conventional microbiologic testing or 16S ribosome genotyping. Instead, accurate identification to the species level of these organisms requires more advanced methods, such as

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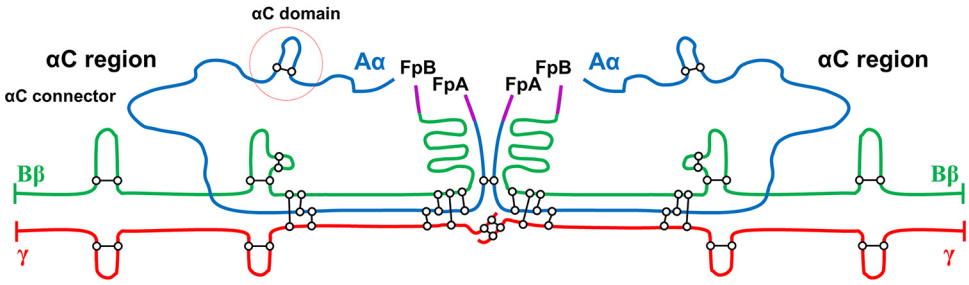

FIG 1 Schematic diagram of the intact fibrinogen dimer. The individual chains, Aα, Bβ, and γ, are blue, green, and red, respectively. Fibrinopeptides A and B (FpA and FpB) are magenta, and the disulfide bonds are shown by black bars. αC domains are consistent with the αC-domain and the flexible αC-connectors.

genome-wide association studies (12), and for this reason, clinical isolates are sometimes identified as "*Streptococcus mitis*/*oralis*" (13–17). These organisms are a leading cause of IE, with mortality ranging from 6% to 30% (18, 19). Despite the increasing importance of endocarditis due to *S. mitis*/*oralis*, especially in view of the high prevalence of multidrug resistance among these strains, relatively little is known about the virulence determinants of *S. mitis*/*oralis*. Our previous studies have identified several surface adhesins of *S. oralis* strain SF100 (formerly identified as *S. mitis*), such as PblA, PblB, and lysin$_{SM1}$, that mediate binding to human platelets and enhance virulence in animal models of IE (20–23). Lysin$_{SM1}$ is encoded by a lysogenic bacteriophage (SM1) and has been shown to have at least two pathogenetic functions. First, lysin is essential for the export of the phage-encoded adhesins, PblA and PblB. In addition, extracellular lysin can bind phosphocholine residues on the bacterial cell wall, where it can mediate bacterial binding to fibrinogen (21). Deletion of the lysin$_{SM1}$ gene in SF100 resulted in significantly lower binding of the organism to fibrinogen and platelets *in vitro* and delayed the onset of platelet aggregation by this strain (20).

Fibrinogen is a 340-kDa glycoprotein comprising three pairs of distinct polypeptide chains (Aα, Bβ, and γ; Fig. 1) that are linked by 29 disulfide bridges (24, 25). It can be polymerized by the hydrolytic catalysis of its terminal ends by thrombin, resulting in fibrin clots or thrombi. Fibrin polymers can be degraded by a proteolytic process known as fibrinolysis, which is tightly controlled by a series of cofactors, inhibitors, and receptors (26–28). The high-affinity binding of plasminogen, a serine protease, to the distal portion of each αC region of fibrinogen Aα chains is the first step of fibrinolysis. Bound plasminogen is then activated to plasmin by cleavage at AA561 by tissue-type plasminogen activator (t-PA), thereby triggering fibrinolysis (29–31).

With a view toward better understanding how lysin$_{SM1}$ interacts with fibrinogen, we identified the specific binding site for lysin$_{SM1}$ within the fibrinogen Aα chain and investigated the effect of this interaction on clotting and fibrinolysis. Our studies indicate that a specific interaction of the binding domain in fibrinogen Aα chain overlaps a region bound by plasminogen. Moreover, binding of this region by lysin$_{SM1}$ inhibits plasmin-mediated fibrinolysis.

RESULTS

Lysin$_{SM1}$ binding to the αC region of fibrinogen Aα. We previously showed that recombinant lysin$_{SM1}$ encoded by bacteriophage SM1 binds to the Aα chain of human fibrinogen and that this interaction enhances the attachment of *S. oralis* SF100 to human platelets (21). The domain of lysin that bound to the Aα chain was contained within the region spanned by amino acid residues 102 to 198 (97 amino acids [AA]) (20). To identify the regions within fibrinogen Aα that bound lysin$_{SM1}$, we expressed and purified recombinant forms of the whole Aα (610 aa; variant 1), N-terminal region (AA1-182; variant 2) and C-terminal region (αC region; AA183-610; variant 3) and examined their binding by lysin$_{102\text{-}198}$ by far-Western blotting (Fig. 2A and B). Lysin$_{102\text{-}198}$ (10 μg) bound to variant 1 and 3, but not variant 2, indicating that the lysin$_{SM1}$ binding

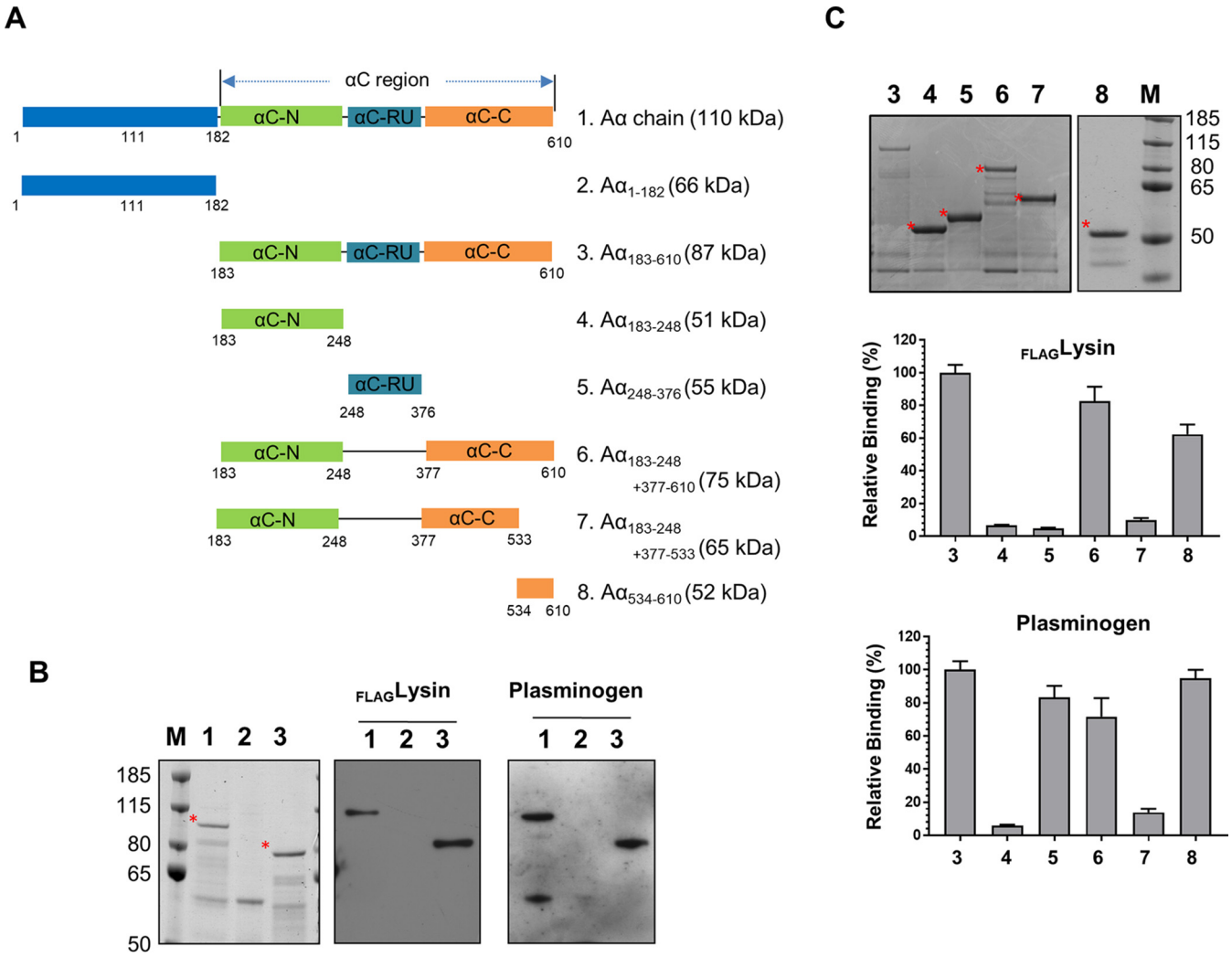

FIG 2 Identification of lysin$_{SM1}$ and plasminogen binding regions on the fibrinogen Aα chain. (A) Schematic diagram of the fibrinogen Aα chain and its recombinant variants. All variants were expressed as MalE fusion proteins. (B) Binding of lysin$_{102\text{-}198}$ (10 μg) or plasminogen (10 μg) to human fibrinogen Aα and Aα variants 2 and 3; analysis by far Western blotting. (C) Binding of lysin$_{102\text{-}198}$ or plasminogen to immobilized recombinant fibrinogen Aα variants 3 to 8 (0.1 μM). Recombinant fibrinogen truncates were separated by SDS-PAGE and stained with Coomassie blue (top). * indicates the expected molecular sizes of recombinant variants. The binding region was identified by measuring lysin$_{102\text{-}198}$ (1 μM; middle) or plasminogen (1 μM; bottom) binding to immobilized fibrinogen truncates. Bound proteins were detected with anti-FLAG or anti-plasminogen monoclonal antibodies.

domain was located on the αC region of Aα chain. To identify the specific binding regions within this domain, several soluble truncated forms of the region fused to MalE were isolated and tested for lysin$_{102\text{-}198}$ binding (Fig. 2C). Recombinant lysin$_{102\text{-}198}$ bound to the variants containing the region spanned by amino acid residues 534 to 610 (variant 8), the C-terminal end of fibrinogen Aα chain.

To better define the plasminogen binding sites on fibrinogen, we examined the binding of recombinant human plasminogen with the above-described fibrinogen Aα chain subdomains, as measured by far-Western blotting. As expected, plasminogen (10 μg) bound the αC region (variants 1 and 3; Fig. 2B). Plasminogen also interacted with an ~60-kDa protein (Fig. 2B, lane 1), which was identified as a fragment of variant 1 by liquid chromatography-tandem mass spectroscopy (LC-MS/MS) (data not shown). In addition, plasminogen bound the variants containing the region spanned by amino acid residues 534 to 610 (variant 8) and 248 to 376 (variant 5; αC-RU) (Fig. 2C), indicating that it has two separate binding sites on the Aα chain (AA183-376 and AA534-610).

We next used overlapping 19-amino acid peptides fused with maltose binding protein (MBP) to localize the lysin binding segment between residues 534 and 610 of Fg

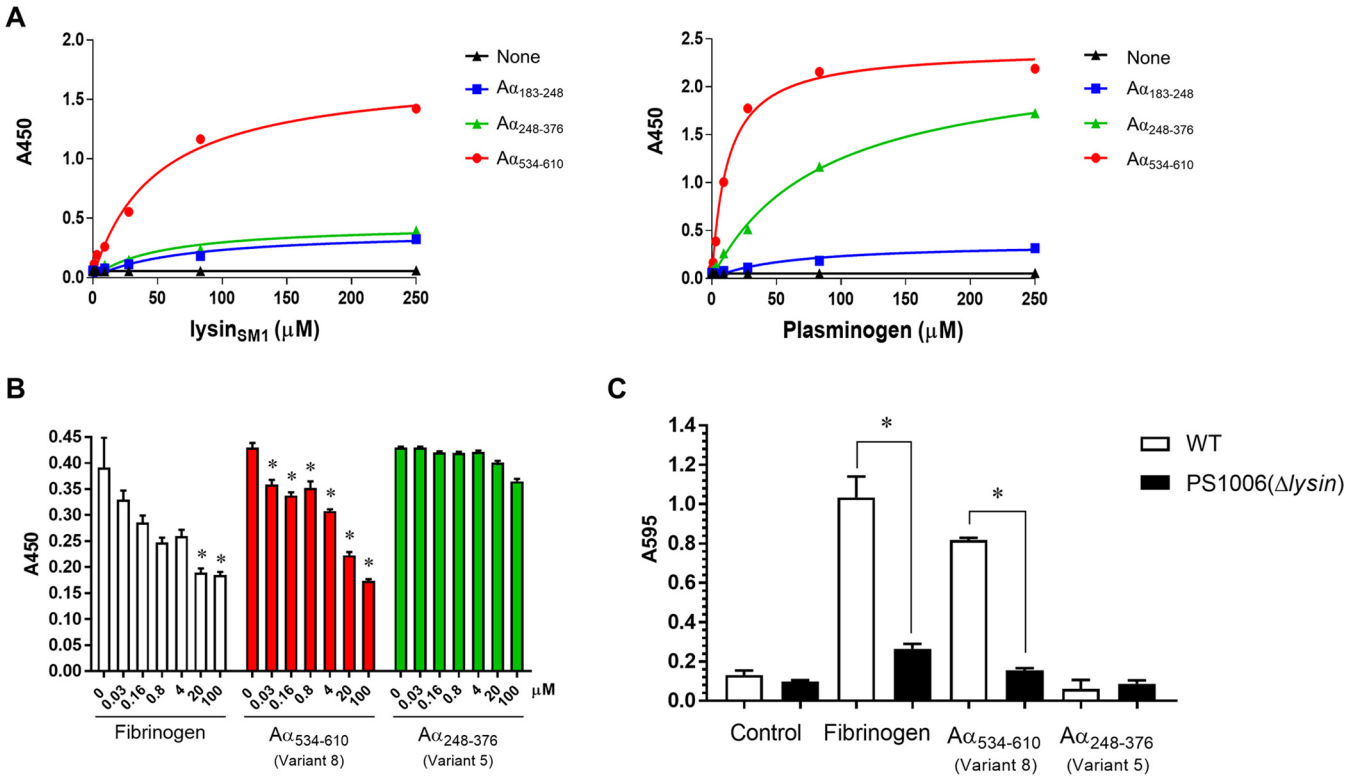

FIG 3 Quantitative analysis of fibrinogen binding by lysin$_{SM1}$, plasminogen, and streptococci. (A) Binding of $_{FLAG}$lysin$_{102-198}$ (left) and plasminogen (right) to immobilized fibrinogen Aα variants 4, 5, or 8 (0.1 μM), as measured by ELISA, using either anti-FLAG or anti-plasminogen antibody. (B) Inhibition of lysin$_{SM1}$ binding to immobilized human fibrinogen, recombinant fibrinogen Aα variant 8, or variant 5. *, $P < 0.05$ compared with the untreated group. (C) Relative binding of *S. oralis* SF100 WT or its isogenic Δ*lysin* mutant (PS1006) to immobilized fibrinogen or fibrinogen Aα variants 8 or 5. Bacteria (10^9 CFU/ml) were incubated with immobilized fibrinogen or fibrinogen Aα variant 8 or 5 (0.1 μM). Bound bacteria were stained with 0.1% crystal violet and measured for optical density at 595 nm. Bars indicate the means (±SD) of triplicate results from a representative experiment. *, $P < 0.05$ compared with WT.

Aα (Fig. S1). Lysin$_{SM1}$ was shown to bind variants 8, 9, 10, and 12, but not variant 11 or 13, indicating that the binding peptide of the fibrinogen Aα chain is localized to AA572-590 (AGSEADHEGTHSTKRGHAK). To further demonstrate that lysin$_{SM1}$ binding is specific, we made targeted point mutations within the lysin$_{SM1}$ binding region (AA102-198) and assessed binding to the Fg Aα chain (Fig. S2). Of the four substitutions tested individually, both H111A and D188A were markedly reduced in binding to MalE:A$\alpha_{534-610}$. These findings indicate that lysin$_{SM1}$ binding to Fg Aα is specific and that these are key residues for this interaction.

To directly compare lysin$_{SM1}$ and plasminogen binding to the αC region, equal amounts (0.1 μM) of variants 4 (A$\alpha_{183-248}$), 5 (A$\alpha_{248-376}$), and 8 (A$\alpha_{534-610}$) were immobilized in 96-well plates, and binding of recombinant lysin$_{102-198}$ and purified plasminogen was measured by enzyme-linked immunosorbent assay (ELISA). As expected, lysin$_{SM1}$ bound to variant 8, with binding reaching a plateau at 75 μM and with an apparent K_D of 4.8×10^{-5} M (Fig. 3A). Lysin$_{102-198}$ showed no binding activity with variants 4 and 5. In contrast, plasminogen bound to variant 5 ($K_D = 8.5 \times 10^{-5}$ M), and variant 8 ($K_D = 1.8 \times 10^{-5}$ M; Fig. 3A). To validate these specific interactions, we also examined whether purified fibrinogen, variant 8, or variant 5 could inhibit lysin$_{SM1}$ binding to immobilized fibrinogen. When lysin$_{SM1}$ (1 μg) was coincubated with 0 to 100 μM of these proteins (Fig. 3B), subsequent binding to fibrinogen by lysin$_{SM1}$ was effectively blocked by purified fibrinogen and variant 8, but not variant 5 (Fig. 3B). In addition, we found that plasminogen (0 to 50 μM) inhibited lysin$_{SM1}$ (1 μg) binding to immobilized fibrinogen (Fig. S3). These data indicate that the fibrinogen binding sites for both lysin$_{SM1}$ and plasminogen are colocalized within the same domain in the αC region (A$\alpha_{534-610}$) of the fibrinogen Aα chain.

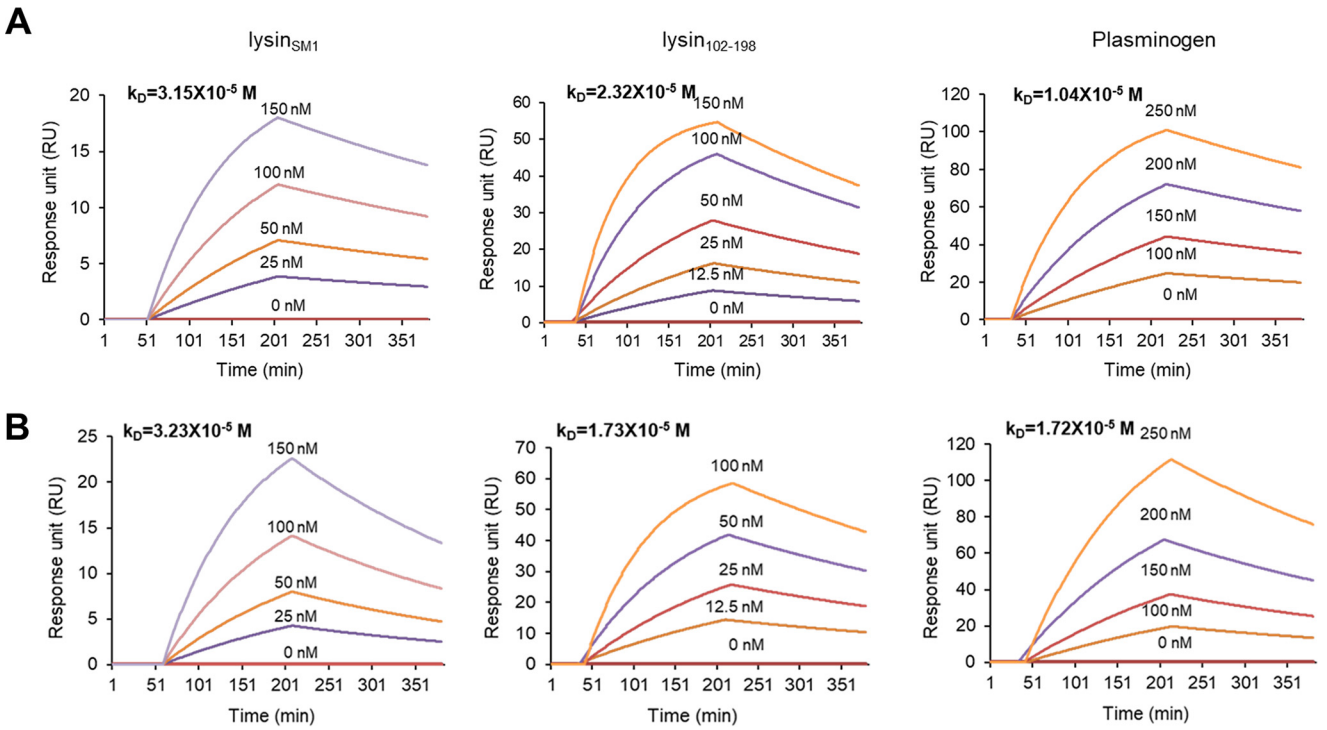

FIG 4 Interaction of plasminogen and lysin$_{102-198}$ with fibrinogen and the fibrinogen αC region. (A and B) Surface plasmon resonance analysis of lysin$_{SM1}$ (left panel), lysin$_{102-198}$ (middle panel), and plasminogen (right panel) binding to (A) fibrinogen and (B) variant 8.

We next assessed the impact of lysin$_{SM1}$ expression on the binding of streptococci to fibrinogen. Wild-type (WT) SF100 and its Δ*lysin* isogenic mutant (PS1006) were compared for binding to immobilized human fibrinogen and recombinant Aα truncates. As shown in Fig. 3C, the WT bound to only fibrinogen and variant 8, but not variant 5. Compared with the WT strain, PS1006 had significantly reduced binding to both fibrinogen ($P = 0.01$) and variant 8 ($P < 0.001$). These findings strongly suggest that lysin$_{SM1}$ on the surface of *S. oralis* mediates binding to the fibrinogen αC region and that the binding domain is located within residues 534 to 610.

Quantitative assessment of lysin$_{SM1}$ binding to the αC region by surface plasmon resonance. We analyzed by surface plasmon resonance (SPR) the binding affinity of lysin$_{SM1,}$ lysin$_{102-198}$, and plasminogen to purified human fibrinogen, by measuring the dissociation constant (K_D), a specific type of equilibrium constant that measures the propensity of dissociation between two components (Fig. 4A). Increasing concentrations of lysin$_{SM1}$ (0 to 150 nM), lysin$_{102-198}$ (0 to 150 nM), and plasminogen (0 to 250 nM) were flowed over immobilized fibrinogen, and the K_D was calculated for each protein. The K_D values of lysin$_{SM1}$, lysin$_{102-198}$, and plasminogen to immobilized fibrinogen were determined to be 3.15×10^{-5}, 2.32×10^{-5}, and 1.04×10^{-5} M, respectively. We next analyzed the binding affinities of lysin$_{SM1}$, lysin$_{102-198}$, and plasminogen to recombinant forms of variant 8 (A$\alpha_{534-610}$) (Fig. 4B). Lysin$_{SM1}$, lysin$_{102-198}$, and plasminogen showed high levels of binding to this peptide, with affinities of 3.23×10^{-5}, 1.73×10^{-5}, and 1.72×10^{-5} M, respectively. These values are within the range reported for other bacterial fibrinogen binding proteins, such as Srr1 of *Streptococcus agalactiae* and SdrG of *Staphylococcus aureus* (32, 33).

Inhibition of plasminogen binding to fibrinogen by lysin$_{SM1}$. Since lysin$_{SM1}$ and plasminogen bound the αC region of fibrinogen Aα with similar affinities, we next determined whether lysin$_{SM1}$, lysin$_{102-198}$, or lysin$_{1-102}$ could competitively inhibit plasminogen binding to immobilized fibrinogen, as measured by ELISA. Immobilized fibrinogen was preincubated with 0 to 100 μM the lysin$_{SM1}$ variants, followed by incubation with 100 nM plasminogen. As shown in Fig. 5A, minimal inhibition was detected for

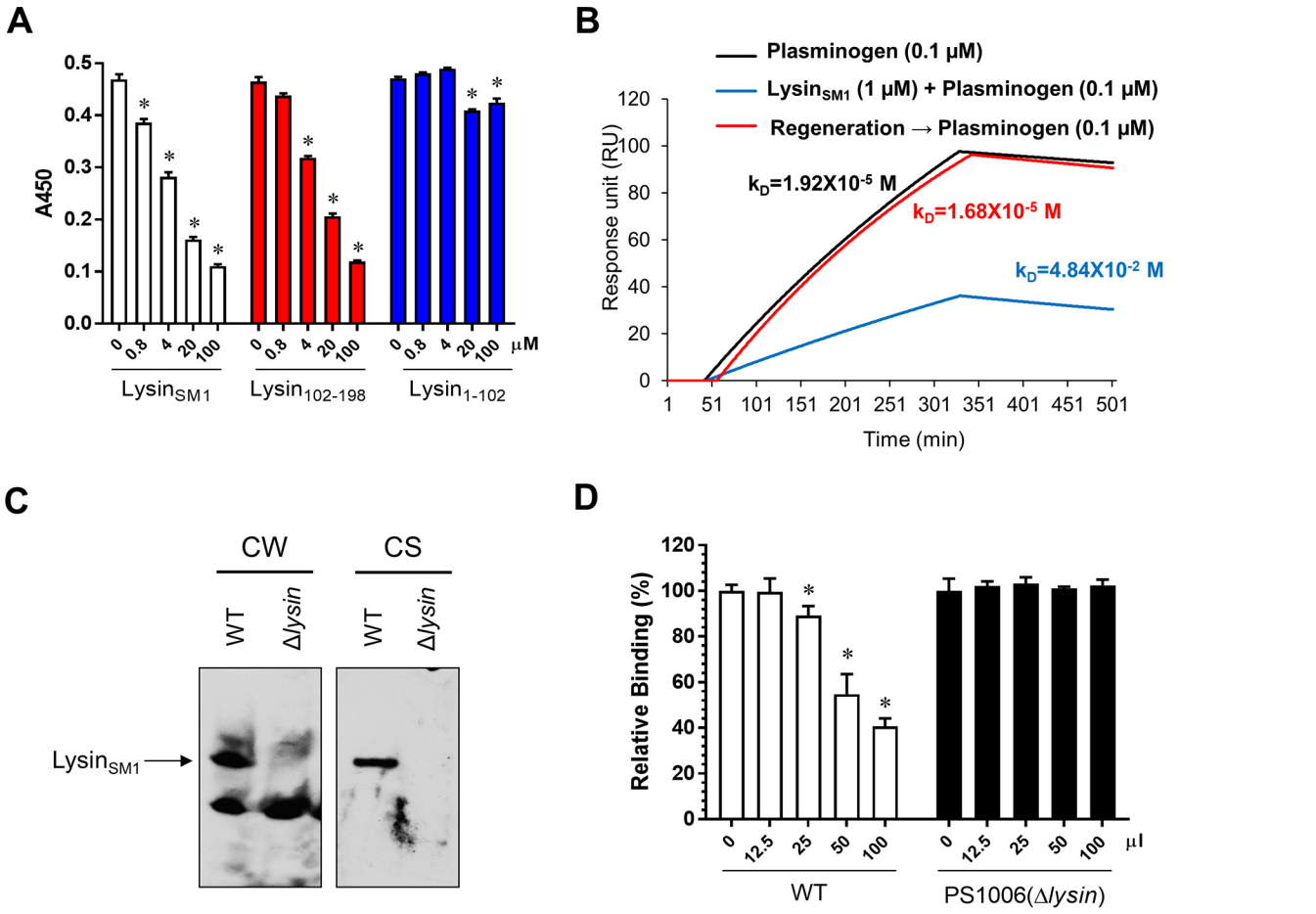

FIG 5 Inhibition of plasminogen binding to fibrinogen by recombinant lysin$_{SM1}$ and secreted lysin$_{SM1}$. (A) Recombinant lysin$_{SM1}$ inhibits plasminogen binding to immobilized fibrinogen. Plasminogen was preincubated with recombinant lysin$_{SM1}$, lysin$_{102-198,}$ or lysin$_{1-102}$ (0, 0.8, 4, 20, and 100 μM) and transferred to 96-well plates containing immobilized fibrinogen (0.1 μM). Bound plasminogen was determined by ELISA with mouse anti-plasminogen antibody. (B) Inhibition of plasminogen binding to fibrinogen by lysin$_{SM1}$ (SPR analysis). Lysin$_{SM1}$ (1 μM) or PBS was streamed over immobilized fibrinogen followed by plasminogen (0.1 μM). A plot of the level of binding (response units) at equilibrium against a concentration of analyte was used to determine the K_D. For the regeneration studies, lysin$_{SM1}$ bound to immobilized fibrinogen was removed by washing with glycine buffer (pH 2.0), streaming with plasminogen (100 nM). (C) Lysin$_{SM1}$ expression in the cell wall extract (CW) or culture supernatant (CS) of *S. oralis* SF100 (WT) and PS1006 (SF100Δ*lysin*) was determined by rabbit anti-lysin$_{SM1}$ IgG. (D) Inhibition of plasminogen binding to immobilized fibrinogen by lysin$_{SM1}$ from *S. oralis* SF100. Wells coated with fibrinogen were preincubated with 0 to 100 μl of concentrated (10×) culture supernatant from WT or PS1006, followed by adding plasminogen (0.1 μM). Bound plasminogen was detected with antiplasminogen antibodies. Bars indicate the means (±SD) of triplicate results from a representative experiment. *, $=P<0.05$ compared with untreated group.

lysin$_{1-102}$, but more than 75% inhibition of plasminogen binding to immobilized fibrinogen was seen with either lysin$_{SM1}$ or lysin$_{102-198,}$ which were significant compared with untreated fibrinogen ($P<0.05$).

To confirm the above-described findings, we also examined by SPR the impact of lysin on plasminogen binding to fibrinogen (Fig. 5B). Lysin$_{SM1}$ (1 μM) was streamed over immobilized fibrinogen followed by the addition of plasminogen (100 nM). Similar to what was seen by ELISA, the affinity (K_D) of plasminogen binding to fibrinogen was 1.92×10^{-5} M. This was reduced to 4.84×10^{-2} M in the presence of lysin$_{SM1}$. We then released the bound lysin from the immobilized fibrinogen by washing the sensor chip surface with a low-pH glycine buffer (pH 2.0). The K_D value of plasminogen binding to immobilized fibrinogen on the chip surface was restored to 1.68×10^{-5} M, indicating that lysin$_{SM1}$ competitively inhibited plasminogen binding to fibrinogen.

We next examined whether native lysin$_{SM1}$ produced by strain SF100 had similar effects on plasminogen binding. As expected (21), lysin$_{SM1}$ was found in cell wall extracts and in the culture supernatants of SF100, but not for PS1006 (Fig. 5C). We

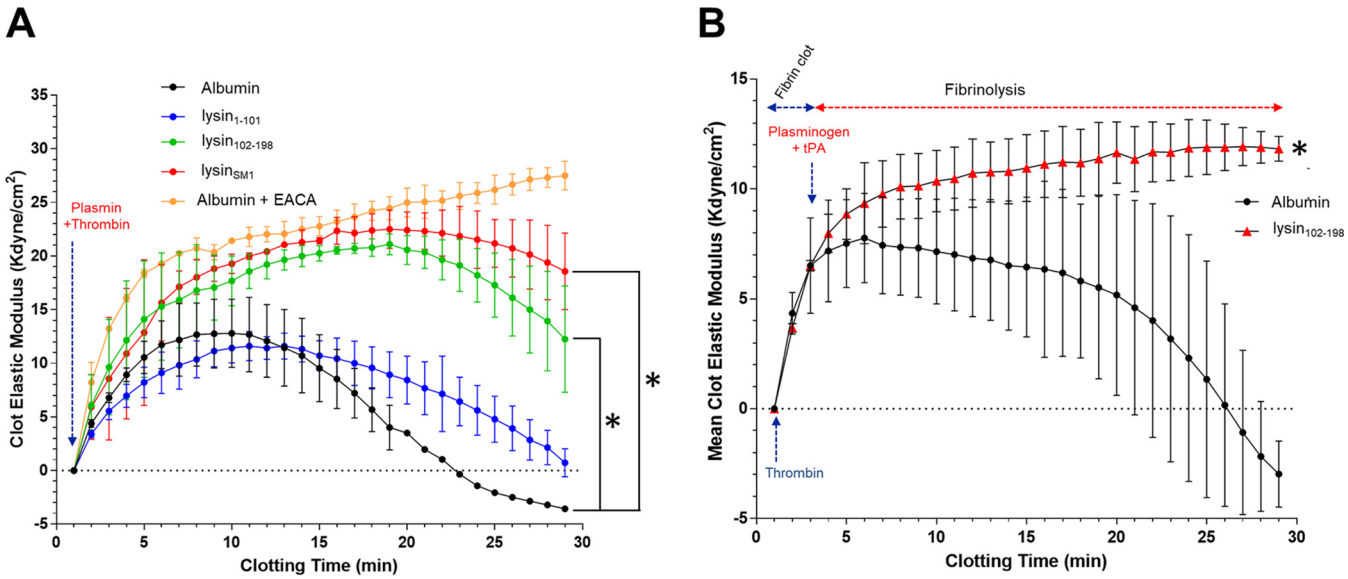

FIG 6 Inhibition of plasmin(ogen)-mediated fibrinolysis by recombinant $lysin_{SM1}$. The impact of $lysin_{SM1}$ on fibrin polymerization and proteolysis was assessed by thromboelastography (TEG). Human fibrinogen (2 mg/ml) and 13.7 μM $lysin_{SM1}$, $lysin_{102\text{-}198}$, or $lysin_{1\text{-}101}$ or albumin (13.7 μM) in HEPES buffer (pH 7.4) were preincubated for 30 min at 37°C. (A and B) Each mixture was transferred to a TEG cup, followed by adding (A) thrombin (1 IU/ml) and plasmin (4 μg/ml) or (B) thrombin (1 IU/ml), tPA (0.5 μg/ml), and plasminogen (4 μg/ml). The clot elastic modulus ($n = 2$) was recorded once per minute for 30 min. *, $P < 0.05$ compared with albumin-treated group.

detected about 0.45 $\pm$ 0.036 μg/ml of $lysin_{SM1}$ in the culture supernatant of SF100, as measured by ELISA. To assess the impact of $lysin_{SM1}$ on plasminogen binding to immobilized fibrinogen, we pretreated fibrinogen-coated wells with 0 to 100 μl of supernatants collected from WT or PS1006 cultures, followed by incubation with plasminogen. As was seen with recombinant $lysin_{SM1}$, the supernatant from WT SF100 significantly inhibited plasminogen binding ($P < 0.006$ for volumes above 12.5 μl), but supernatants from PS1006 had no effect (Fig. 5D).

Inhibition of fibrinolysis by blocking plasminogen binding to the αC region by $lysin_{SM1}$. Fibrinolysis requires the binding of plasminogen to the C-terminal region of fibrinogen or fibrin, followed by its cleavage by tissue plasminogen activator (tPA), thereby generating the active protease plasmin (34, 35). To assess the impact of $lysin_{SM1}$ on fibrinolysis, we examined the impact of lysin on clot formation and dissolution *in vitro*, using thromboelastography (TEG). Fibrinogen was preincubated with 13.7 μM albumin (as a control), followed by adding thrombin (to activate fibrin formation and polymerization) and plasmin (to initiate fibrinolysis). Clotting was detectable within 2 min, as indicated by an increase in the elastic modulus, and peaked at 10 min. This was followed by a decline in the modulus, indicating ongoing fibrinolysis, which reached lower than zero shear modulus strength (kdyne/cm^2) after 24 min (Fig. 6A). Fibrinolysis was completely blocked by addition of epsilon-aminocaproic acid (EACA; 130 μg/ml), a standard lysine analogue used to competitively inhibit plasmin-induced fibrinolysis (36). When fibrinogen was preincubated with $lysin_{SM1}$, clotting reached significantly higher levels at 10 min and peaked at 15 min. These high levels of clotting and resistance to proteolysis were sustained even at 30 min postexposure to thrombin and plasmin ($P < 0.001$).

The above-described studies demonstrated that $lysin_{SM1}$ could inhibit fibrinolysis. To determine whether this was due to the competitive inhibition of plasmin binding to the αC region, fibrinogen was preincubated with $lysin_{102\text{-}198}$ or $lysin_{1\text{-}101}$. When tested by TEG, preincubation with $lysin_{1\text{-}101}$ had a minimal effect on plasmin-induced fibrinolysis. In contrast, $lysin_{102\text{-}198}$ reduced fibrinolysis ($P < 0.001$) to levels that were comparable to those seen with $lysin_{SM1}$, indicating that the inhibition of fibrinolysis by $lysin_{SM1}$ is due to its blocking of plasmin binding.

In vivo, fibrinolysis requires the conversion of plasminogen to plasmin by tPA.

However, tPA can only activate plasminogen that is bound to fibrinogen. We therefore examined whether lysin$_{SM1}$ binding to fibrinogen could inhibit fibrinolysis induced by tPA. Fibrinogen was mixed with lysin$_{102-198}$ or 13.7 μM albumin (as a control) and incubated for 4 min with thrombin, followed by the addition of plasminogen and tPA. As expected, tPA induced extensive clot lysis when mixed with plasminogen alone (Fig. 6B). However, tPA failed to induce lysis in the presence of plasminogen and lysin$_{102-198}$ ($P < 0.001$). Since tPA can only activate plasminogen bound to fibrinogen, these data indicate that the blocking of tPA-mediated fibrinolysis by lysin$_{SM1}$ is due to the inhibition of plasminogen binding to the fibrinogen αC region, such that tPA can no longer generate plasmin and clot lysis.

DISCUSSION

Lysin$_{SM1}$ is a key adhesin of *S. oralis* SF100, mediating bacterial binding to platelets *in vitro* through its interaction with fibrinogen on the platelet surface. However, it was unknown which regions of fibrinogen were bound by lysin$_{SM1}$, in part because this adhesin has no structural homology to other known fibrinogen binding proteins, as measured by amino acid sequence alignment (T-Coffee) and protein structure homology-modeling (SWISS-MODEL) (37, 38). Our studies indicate that lysin$_{SM1}$ binds residues 534 to 610 of the fibrinogen Aα chain. This differs from other known fibrinogen binding proteins of other bacteria, such as staphylococcal ClfB and Srr1 and Srr2 of *Streptococcus agalactiae* (both bind AA283-410 of the Aα chain), staphylococcal SdrG (AA1-25 of the β chain), and staphylococcal ClfA, FnBPA, and FnBPB (AA6-20 of the γ chain) (20, 21, 33, 39–41). Staphylococcal bone sialoprotein-binding protein (Bbp) binds the same region (AA561-575) of the Aα chain as lysin$_{SM1}$ (42). However, lysin$_{SM1}$ differs from at least some of these proteins in its impact on clotting. In particular, SdrG of *Staphylococcus epidermidis* inhibits coagulation by binding to the thrombin cleavage sites on fibrinogen (33), and Bbp of *Staphylococcus aureus* has anticoagulant action through an unknown mechanism via binding to AA561-575 (40). In contrast, lysin$_{SM1}$ has no direct effect on clot formation, at least as measured by TEG, but does have strong anti-fibrinolysis effects, by inhibiting the binding of plasminogen to the αC region of fibrinogen Aα chain.

Pathogenic bacteria can produce and secrete activators or inhibitors of fibrinolysis that may impact their survival and dissemination. At least two distinct mechanisms involving plasminogen have been observed. First, plasminogen binding proteins of bacteria, such as streptokinase from group A, C, and G streptococci, staphylokinase of *S. aureus*, and Pla of *Yersinia pestis* (39–44), can bind free circulating plasminogen and convert it to plasmin (43–48). Second, proteins on the surface of bacteria, such as GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and enolase of streptococci, plasminogen-binding protein (PAM) of *Streptococcus pyogenes*, and OspA/C of *Borrelia burgdorferi*, bind plasminogen, which is then converted to plasmin by tPA (49–52). Here, we report a novel mechanism for inhibiting fibrinolysis, in which lysin$_{SM1}$ competitively inhibits plasminogen binding to the fibrinogen A$\alpha_{534-610}$ region, such that it can no longer activate fibrinolysis. We also found that plasminogen bound a second region (AA248-376) within the αC region of fibrinogen. However, the binding affinity for this region was about five times lower than at the primary binding site (AA534-610). This lower affinity would explain our finding that inhibition of fibrinolysis by lysin$_{SM1}$ did not appear to be affected by plasminogen binding to the second binding site.

Our previous studies using an animal model of infective endocarditis demonstrated that loss of lysin$_{SM1}$ expression by SF100 was associated with decreased virulence, as measured by reduced levels of bacteria (CFU/g of tissue) within vegetations on cardiac valves, as well as in kidneys and spleens (21). Part of this reduced virulence is likely due to the loss of fibrinogen-mediated binding to platelets, which is a key step for the initial attachment of bacteria to damaged valve surfaces, as well as for the subsequent formation of infected vegetations. These structures are composed of bacteria embedded in a biofilm containing platelets, fibrinogen, and fibrin (53). Vegetation formation

is thought to protect bacteria from phagocytosis and render these organisms less susceptible to antimicrobials. Moreover, larger vegetations are associated with increased embolization to target organs, such as the kidneys, spleen, and brain (54–56). Fibrinolysis may serve to mitigate vegetation formation, as both *in vitro* and *in vivo* studies have shown that tPA may reduce vegetation size and facilitate antimicrobial therapy (57–60). Thus, an additional mechanism by which $lysin_{SM1}$ may enhance virulence is by blocking plasminogen binding to fibrinogen/fibrin within vegetations, thereby inhibiting tPA activation and vegetation lysis.

Although these studies examined a single strain of *S. oralis*, our findings are likely to be applicable to a broad range of organisms. Metagenomic studies by Willner et al. indicate that bacteriophage SM1 is highly prevalent in the oral microbiome and that it is the most common bacteriophage of Gram-positive organisms in the oral cavity (61). Moreover, the $lysin_{SM1}$ gene was among the open reading frames (ORFs) of SM1 most frequently detected. This group also examined the published salivary metagenomes from nine individuals, and all were found to contain *pblA* and *pblB*, two phage morphogenesis genes adjacent to *lysin* on the SM1 genome. More recently, metagenomic studies of salivary specimens from children detected bacteriophage SM1 in 29 of 30 individuals (62). In addition, lysin is among the most commonly expressed genes of streptococcal bacteriophages within the oral microbiome. These findings strongly indicate that SM1 or similar bacteriophages encoding a $lysin_{SM1}$ homolog are highly prevalent in the oral microbiome (63). Our own searches for homologs of $lysin_{SM1}$ indicate that numerous strains of not only *S. oralis*, but also and *S. mitis* and *Streptococcus pneumoniae* (data not shown) encode such homologs, indicating that $lysin_{SM1}$ may be widely prevalent in a variety of streptococcal species.

In summary, we propose that expression of streptococcal phage $lysin_{SM1}$ impacts the pathogenesis of infective endocarditis in the following manner: (i) damage of the endocardium by inflammation or hemodynamic trauma induces the deposition of platelets, fibrinogen, and fibrin polymerization onto the valve surface (Fig. 7A); (ii) attachment of streptococci encoding $lysin_{SM1}$, such as *S. oralis* SF100, to the altered surface. This initiates endocardial infection and attachment of free or cell wall $lysin_{SM1}$ to the αC region of fibrinogen, thereby blocking the binding of circulating plasminogen and tPA (Fig. 7B); (iii) the further deposition and polymerization of fibrinogen onto the infected endocardium along with the proliferation of bacteria on the valve surface, resulting in extensive vegetation formation (Fig. 7C).

MATERIALS AND METHODS

Strains and growth conditions. The bacteria and plasmids used in this study are listed in Table S1. Strain SF100 is an endocarditis-associated clinical isolate (64). Originally identified as *S. mitis* by conventional clinical laboratory methods, we have recently sequenced the complete genome of this strain. BLAST analysis (v2.7.1) was carried out to identify to which species it shows similarity. The average nucleotide identity (ANI) values used to compare the genome of SF100 with *S. mitis* and *S. oralis* were determined using the OrthoANIu algorithm (https://www.ezbiocloud.net/tools/ani) (65). In addition, the whole-genome sequences were aligned with 120 bacterial marker genes, using GTDB-Tk (v1.3.0) (66), and the best-fit model was selected using ModelTest-NG (v0.1.6) (67). A phylogenetic tree was constructed for the PROTGAMMALGF model using RAxML (v8.2.12), including bootstrap analysis based on 100 replicates (68). BLAST analysis of the complete SF100 genome found the highest similarity with *S. oralis* ATCC 35037 (GenBank accession no. LR134336), with ANI values of 95.5% and 86.2%, respectively, for *S. oralis* ATCC 35037 and *S. mitis* NCTC12261 (CP028414). Because an ANI cutoff of 95 to 96% is used for species definition (65), these results indicated that SF100 should be classified as a strain of *S. oralis*. In the phylogenetic tree analysis (Fig. S4), the *S. mitis* and *S. oralis* groups were clearly separated, and SF100 clustered with *S. oralis* strains, further indicating that SF100 is a member of this species.

SF100 was grown in Todd-Hewitt broth (Difco, Franklin Lakes, NJ) supplemented with 0.5% yeast extract (THY). *Escherichia coli* strains were grown at 37°C under aeration in Luria broth (LB, Difco). Appropriate concentrations of antibiotics were added to the medium, as required.

Cloning and expression of fibrinogen Aα and its variants. cDNA encoding the Aα chain of human fibrinogen was generously provided by Susan Lord (University of North Carolina at Chapel Hill) (69–71). Full-length and truncates of the Aα chain were cloned into pMAL-C2X (New England Biolabs, Ipswich, MA) with specific primer sets (Table S2) to express maltose binding protein MalE-tagged versions of variants. All recombinant proteins were purified by affinity chromatography with amylose resin according to the manufacturer's instructions (New England Biolabs).

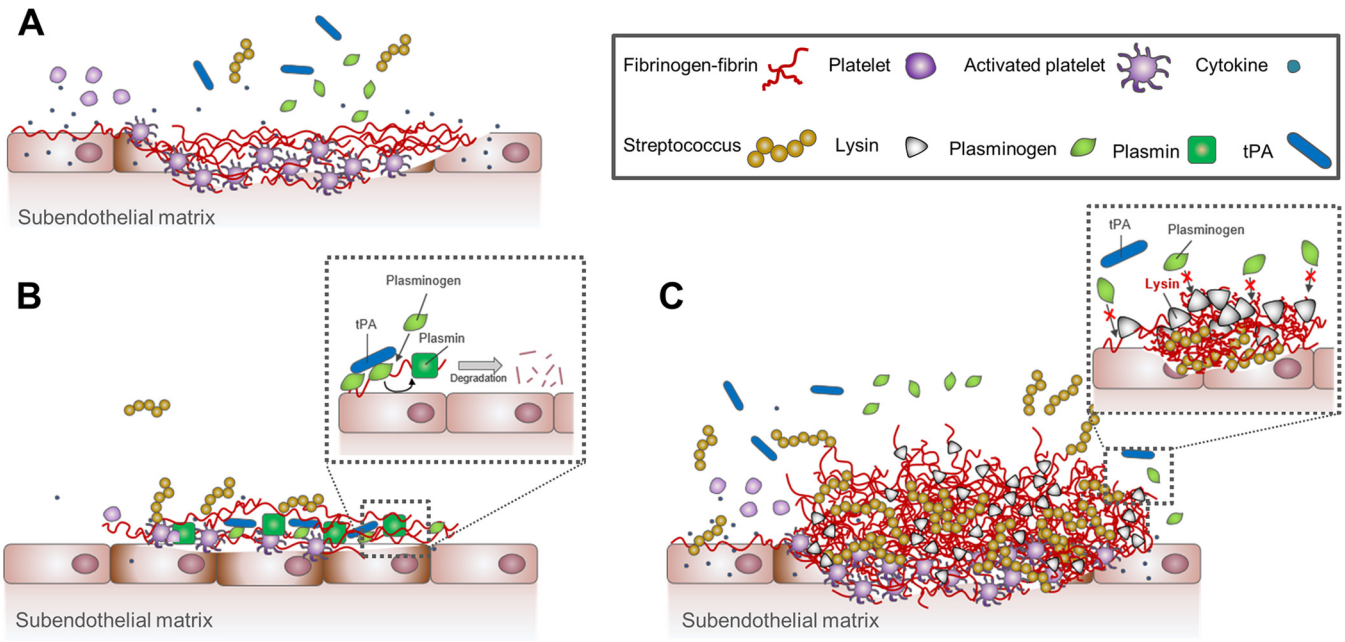

FIG 7 Model of the role of lysin$_{SM1}$ during streptococcal proliferation on the surface of a damaged valve. (A) Damaged endocardium becomes covered with platelets and extracellular matrix proteins, including fibrinogen. Thrombin converts fibrinogen to fibrin polymers. (B) Streptococci circulating in the bloodstream bind to fibrin and immobilized platelets on the endovascular surface. Plasminogen binds to the αC region of the fibrinogen Aα chain, followed by recruitment of tPA, which converts plasminogen to plasmin. Plasmin degrades the fibrin clot, thereby disrupting the vegetation and releasing streptococci. (C) Exported lysin$_{SM1}$ binds to the αC region of the fibrinogen Aα chain, thereby blocking the binding of plasminogen and inhibiting fibrinolysis.

Site-directed mutagenesis. Point mutations of lysin$_{SM1}$ were generated using a Muta-Direct site directed mutagenesis kit (Intron, Inc., Seoul, South Korea) and pET28$_{FLAG}$-lysin$_{SM1}$ as the template plasmid. The resulting plasmids were screened for the expected mutations by DNA sequencing (Macrogen, Inc., Seoul, South Korea). After the correct sequences were confirmed, plasmids were introduced into *E. coli* BL21(DE3) to express and purify recombinant forms of mutated lysin$_{SM1}$.

Analysis of lysin$_{SM1}$, lysin$_{102\text{-}198}$, or plasminogen binding to fibrinogen and its variants by far-Western blotting. Purified human fibrinogen (Haematologic Technologies, Essex Junction, VT) and recombinant fibrinogen Aα variants were separated by electrophoresis through 3 to 8% NuPAGE Tris-acetate gels (Invitrogen, Waltham, MA) and transferred onto polyvinylidene difluoride (PVDF) membrane (Merck Millipore, Burlington, MA). The membranes were blocked with a casein-based solution (Roche, Basel, Switzerland) at room temperature (RT) for 1 h and then incubated for 1 h with FLAG-tagged lysin$_{SM1}$, FLAG-tagged lysin$_{102\text{-}198}$, or purified plasminogen (1 μM) in phosphate-buffered saline (PBS)-0.05% Tween 20 (PBS-T). The membranes were then washed three times for 15 min in PBS-T, and bound protein was detected with mouse anti-FLAG antibody (1:4,000; Sigma-Aldrich, St. Louis, MO) or rabbit anti-plasminogen antibody (1:3,000; Abcam, Cambridge, UK).

Analysis of lysin$_{SM1}$, lysin$_{102\text{-}198}$, or plasminogen binding to fibrinogen and its variants by enzyme-linked immunosorbent assay (ELISA). Purified fibrinogen or recombinant fibrinogen variants (0.1 μM in PBS) were immobilized overnight in 96-well microtiter plates at 4°C. The wells were blocked with 300 μl of a casein-based solution for 1 h at room temperature (72). The plates were washed three times with PBS-T, and lysin$_{SM1}$, lysin$_{102\text{-}198}$, or plasminogen in PBS-T was added over a range of concentrations for 1 h. The plates were incubated for 1 h at 37°C, washed with PBS-T to remove unbound protein, and incubated with mouse anti-FLAG antibodies (1:4,000) or rabbit anti-plasminogen antibodies (1:3,000) in PBS-T for 1 h at 37°C. Wells were washed and incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (1:5,000; Sigma-Aldrich) or goat anti-rabbit IgG (1:5,000; Sigma-Aldrich) in PBS-T for 1 h at 37°C. For some studies, wells containing immobilized fibrinogen were pretreated with lysin$_{SM1}$, lysin$_{102\text{-}198}$, lysin$_{1\text{-}102}$, truncated recombinant fibrinogen variant A$\alpha_{534\text{-}610}$ or A$\alpha_{248\text{-}376}$, or concentrated supernatants collected from *S. oralis* SF100 (WT) and its isogenic mutant (PS1006 = SF100Δ*lys*) (21), followed by washing prior to the addition of FLAG-tagged lysin$_{SM1}$. Levels of binding were assessed by absorbance at 450 nm, using 3,3′,5,5′-tetramethylbenzidine as the chromogenic substrate. Binding data were analyzed with GraphPad Prism v7.0, using a nonlinear regression curve fit and an on-site total binding equation to estimate the equilibrium binding constant (K_D) for respective conditions.

Binding of *S. oralis* to immobilized fibrinogen and recombinant proteins. Overnight cultures of *S. oralis* SF100 and PS1006 were harvested by centrifugation and suspended in PBS (final concentration, 10^9 CFU/ml). Purified fibrinogen or recombinant truncated fibrinogen variants (0.1 μM) were immobilized in 96-well microtiter plates and then incubated with 100 μl of bacterial suspension for 30 min at 37°C. Unbound bacteria were removed from the plates by washing with PBS, and the

number of bound bacteria was determined by staining with crystal violet (0.5% vol/vol) for 1 min, as described previously (32).

Surface plasmon resonance (SPR) spectroscopy. SPR spectroscopy was performed using a Reichert-4 SR7500DC system (Reichert Technologies, Munich, Germany). Purified human fibrinogen (0.1 μM) in sodium acetate buffer (pH 5.5) was covalently immobilized on a plain gold surface polyethylene glycol (PEG) sensor chip. Increasing concentrations (range, 0 to 250 μM) of lysin$_{SM1}$, lysin$_{102-198}$, or plasminogen in PBS were flowed over fibrinogen at a rate of 30 μl/min with 3 min association and dissociation times. The sensorgram data were subtracted from the corresponding data from the reference flow cell and analyzed using Scrubber2 software (Reichert Technologies). A plot of the level of binding (response units) at equilibrium against a concentration of analyte was used to determine the K_D.

Analysis of lysin$_{SM1}$ expression by Western blotting. *S. oralis* SF100 and PS1006 were harvested by centrifugation of liquid cultures at an A_{600} of 0.8, and the pellet was lysed with 6 M urea. The culture supernatants were concentrated by centrifugation with Amicon Ultra-50 units (Merck Millipore). The samples were separated by SDS-PAGE with 3 to 8% Tris-acetate gels (Invitrogen) under reducing conditions and then were transferred to nitrocellulose membranes. After blocking, the membranes were incubated with rabbit anti-lysin$_{SM1}$ IgG (1:3,000), followed by incubation with HRP conjugated goat anti-rabbit IgG (1:5,000).

Analysis of fibrinolysis using thromboelastography (TEG). Fibrinogen polymerization and fibrinolysis were assessed by thromboelastography, using a Hemodyne hemostasis analysis system (Haemonetics, Niles, IL) as described previously (73). This technique measures clot stiffness ("elastic modulus") over time as an indicator of clot formation or lysis. Human fibrinogen (2 mg/ml) and 13.7 μM lysin$_{SM1}$, lysin$_{102-198}$, or lysin$_{1-101}$ in HEPES buffer (pH 7.4) were preincubated for 30 min at 37°C and transferred to a TEG cup at 37°C, followed by addition of thrombin (1 IU/ml) and plasmin (4 μg/ml) or thrombin (1 IU/ml), tPA (0.5 μg/ml), and plasminogen (4 μg/ml). The clot elastic modulus ($n = 2$) was recorded once per minute for 30 min.

Statistical analysis. Data expressed as means ± standard deviations (SD) were compared for statistical significance using the unpaired t test with Prism v7.0 (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered to be statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 0.9 MB.

FIG S2, TIF file, 0.6 MB.

FIG S3, TIF file, 0.5 MB.

FIG S4, TIF file, 0.7 MB.

TABLE S1, TIF file, 1.6 MB.

TABLE S2, TIF file, 1.1 MB.

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