

Characterization of Streptococcal Platelet-Activating Factor Acetylhydrolase Variants That Are Involved in Innate Immune Evasion

Guanghui Liu, Mengyao Liu, Gang Xie, Benfang Lei

Department of Immunology and Infectious Diseases, Montana State University, Bozeman, Montana, USA

Human pathogen group A streptococcus (GAS) has developed mechanisms to subvert innate immunity. We recently reported that the secreted esterase produced by serotype M1 GAS (SsE^{M1}) reduces neutrophil recruitment by targeting platelet-activating factor (PAF). SsE^{M1} and SsE produced by serotype M28 GAS (SsE^{M28}) have a 37% sequence difference. This study aims at determining whether SsE^{M28} is also a PAF acetylhydrolase and participates in innate immune evasion. We also examined whether SsE evolved to target PAF by characterizing the PAF acetylhydrolase (PAF-AH) activity and substrate specificity of SsE^{M1}, SsE^{M28}, SeE, the SsE homologue in *Streptococcus equi*, and human plasma PAF-AH (hpPAF-AH). PAF incubated with SsE^{M28} or SeE was converted into lyso-PAF. SsE^{M1} and SsE^{M28} had k_{cat} values of 373 s⁻¹ and 467 s⁻¹, respectively, that were ≥ 30 -fold greater than that of hpPAF-AH (12 s⁻¹). The comparison of SsE^{M1}, SsE^{M28}, and hpPAF-AH in k_{cat} and K_m in hydrolyzing triglycerides, acetyl esters, and PAF indicates that the SsE proteins are more potent hydrolases against PAF and have high affinity for PAF. SsE^{M28} possesses much lower esterase activities against triglycerides and other esters than SsE^{M1} but have similar potency with SsE^{M1} in PAF hydrolysis. Deletion of *sse*^{M28} in a *covS* deletion mutant of GAS increased neutrophil recruitment and reduced skin infection, whereas in *trans* expression of SsE^{M28} in GAS reduced neutrophil infiltration and increased skin invasion in subcutaneous infection of mice. These results suggest that the SsE proteins evolved to target PAF for enhancing innate immune evasion and skin invasion.

As a strict human pathogen, group A streptococcus (GAS) causes a broad spectrum of illness ranging from relatively mild pharyngitis to severe infections, such as necrotizing fasciitis and streptococcal toxic shock syndrome (1). The global burden of GAS diseases causes more than 700 million cases of mild, noninvasive infections each year, and at least 517,000 deaths each year due to severe infections (2). The capacity of GAS to cause infections partly depends on its evasion of neutrophil responses. This innate immune evasion is achieved by the combination of different mechanisms through the action of extracellular virulent factors, including the surface protein M protein, the hyaluronic acid capsule, IL-8/CXC chemokine peptidase SpyCEP, C5a peptidase ScpA, the secreted esterase (SsE), streptolysins S (SLS) and O (SLO), and secreted Mac/IdeS protein. ScpA, SpyCEP, and SsE reduce neutrophil ingress into infection sites (3–8). The M protein and hyaluronic acid capsule confer GAS resistance to phagocytosis by neutrophils (9, 10). Mac/IdeS inhibits opsonophagocytosis by interfering IgG-Fcγ receptor interaction (11, 12). In addition, SLS and SLO cause necrosis and apoptosis of neutrophils (13, 14). Most of these virulence factors are negatively regulated by the two-component regulatory system *covRS* (also known as *csrRS*) (15, 16), and the release of the *covRS* suppression of the virulence factors critically contributes to the severe evasion of neutrophil responses by and hypervirulence of GAS with naturally selected *covS*-null mutations or deletion (17).

Our laboratory has been studying the molecular mechanisms of GAS evasion of innate immunity. We recently found that SsE plays a critical role in GAS inhibition of neutrophil recruitment and functions in part by hydrolyzing platelet-activating factor (PAF) (8, 17). PAF is a potent lipid mediator and is involved in platelet aggregation, inflammation, neutrophil activation, and allergic response (18–22). In addition, PAF has been implicated as a

chemoattractant in recruitment of eosinophils, neutrophils, and monocytes (22). The potent biological functions of PAF are regulated by PAF-acetylhydrolases (PAF-AHs) (23–26), which inactivate PAF by removing the acetyl ester group at the *sn*-2 position of PAF. Known mammal PAF-AHs include the secreted or plasma, intracellular type I, and intracellular type II PAF-AHs (23–26). These PAF-AHs belong to different phospholipase A₂ groups (27). Group VII includes the plasma and intracellular type II PAF-AHs, and group VIII has the type I PAF-AHs. Although the type I PAF-AHs are specific for PAF, the plasma and type II PAF-AHs have broader substrate specificity (28).

PAF-AH activity has been also detected in bacteria and fungi. An intracellular yeast group VII PAF-AH enhances the viability of yeast under oxidative stress (29). The spirochete *Leptospira interrogans* produces a PAF-AH (30). An apparently intracellular esterase Est13 from an earthworm gut-associated microorganism inhibits PAF-induced platelet aggregation (31). Both *L. interrogans* PAF-AH and Est13 share sequence homology with the α1 subunit of the type I intracellular mammalian PAF-AH. The function of these bacterial PAF-AHs is not known. In contrast to these yeast and bacterial PAF-AHs, PAF-AH produced by serotype M1 GAS (SsE^{M1}) is a secreted protein (32). This SsE^{M1} protein is a

Received 28 March 2013 Returned for modification 22 April 2013

Accepted 5 June 2013

Published ahead of print 17 June 2013

Editor: R. P. Morrison

Address correspondence to Benfang Lei, blei@montana.edu.

Copyright © 2013, American Society for Microbiology. All Rights Reserved.

doi:10.1128/IAI.00398-13

protective antigen and virulence factor and contributes to innate immune evasion partly by hydrolyzing PAF (8, 32, 33).

There are two SsE variant complexes among GAS isolates. Complex I includes the SsE proteins that are produced by serotypes M1, M2, M3, M5, M6, M12, and M18 GAS, and complex II has the SsE proteins that are produced by M4, M28, and M49. The SsE proteins within each complex share >98% identity in amino acid sequence but have ca. 37% sequence variation between the complexes (32). It is not known whether SsE within complex II also has PAF-AH activity and is involved in innate immune evasion. The enzymatic parameters and substrate specificity of SsE^{M1} have not been determined. To address this question, we prepared SsE of serotype M28 GAS (SsE^{M28}) and characterized its esterase and PAF-AH activities in comparison with SsE^{M1} and human plasma PAF-AH (hpPAF-AH) and its contribution to GAS inhibition of neutrophil recruitment. We found that SsE^{M28} is a potent PAF-AH and has much lower esterase activities against tested esters than SsE^{M1} and that SsE^{M28} contributes to GAS inhibition of neutrophil recruitment and skin invasion.

MATERIALS AND METHODS

Materials. Vinyl propionate, vinyl butyrate, triacetyl glycerol, tripropionyl glycerol, tributyril glycerol, trioctanoyl glycerol, and diisopropyl fluorophosphate (DFP) were purchased from Sigma (St. Louis, MO). Vinyl acetate was purchased from Fisher Scientific (Fair Lawn, NJ). PAF C-16 (1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine), lyso-PAF C-16 (1-O-hexadecyl-*sn*-glycero-3-phosphocholine), recombinant hpPAF-AH, and 2-thio-PAF were purchased from Cayman Chemical (Ann Arbor, MI). [Acetyl-³H]PAF with a specific radioactivity of 27 Ci/mmol was purchased from Perkin-Elmer, Inc. (Boston, MA).

Gene cloning and SsE^{M28} expression and purification. The fragment of the *sse*^{M28} gene encoding the mature SsE^{M28} (amino acid residues 27 to 333) was PCR amplified from serotype M28 GAS strain BAA1064 (also called MGAS6180), which was obtained from ATCC, using the primers 5'-ACCATGGATGCCAGTCTCTAGATCTTG-3' and 5'-AGAATCTTGTAAAGCTTTTGAATCG-3'. The underlined sequences were added to introduce NcoI and EcoRI restriction sites, respectively. The PCR product obtained was cloned into pET-His2 (34) at the NcoI and EcoRI sites. Recombinant SsE^{M28} was expressed and purified from *E. coli* strain BL21(DE3) that contained the cloned *sse*^{M28} gene. The bacteria were grown in 6 liters of Luria-Bertani broth supplemented with 100 mg of ampicillin/liter at 37°C. When the optical density at 600 nm (OD₆₀₀) of the culture was ~0.5, the final concentration of 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added to induce SsE^{M28} production. After 10 h of induction, bacteria were harvested by centrifugation. The bacterial pellet obtained was suspended in 80 ml of 20 mM Tris-HCl (pH 8.0), sonicated on ice for 20 min, and centrifuged. The lysate was adjusted to 0.5 M NaCl and loaded on a Ni-nitrilotriacetic acid agarose column (2.5 by 3 cm). The column was washed with 50 ml of 20 mM Tris-HCl containing 0.5 M NaCl and eluted with 70-ml gradient of 0 to 100 mM imidazole. Fractions containing SsE^{M28} were pooled and dialyzed against 3 liters of 20 mM Tris-HCl, and the dialyzed sample was loaded onto a DEAE-Sepharose column (1.5 by 7 cm). The column was washed with 20 ml of 20 mM Tris-HCl and eluted with a 90-ml gradient of 0 to 200 mM NaCl, and fractions containing SsE^{M28} with 95% purity were pooled, dialyzed against 20 mM Tris-HCl, and concentrated using GE Healthcare Vivaspin 20 filtration devices.

Recombinant SsE protein of serotype M1 GAS (SsE^{M1}) and SeE, the homologue of SsE in *Streptococcus equi*, were prepared as previously described (32, 35). Protein concentrations were determined using the modified Lowry protein assay kit from Pierce with bovine serum albumin as a standard.

Assays for PAF-AH activity. SsE-catalyzed hydrolysis of PAF was monitored by thin-layer chromatography (TLC), a colorimetric assay us-

ing 2-thio-PAF (8), and the standard assay using [acetyl-³H]PAF (36). In the TLC assay, PAF (0.29 mM) was mixed with 7 μM SsE^{M28} in 20 μl of 20 mM Tris-HCl (pH 8.0) or the buffer (negative control reaction), and the reactions were stopped by adding 20 μl of acetonitrile containing 1% formic acid after 20 min incubation at room temperature. Then, 4 μl of the reaction samples, lyso-PAF, and PAF/lyso-PAF mixtures were spotted on a Whatman K6F Silica Gel 60Å TLC plate, and these compounds were resolved using a chloroform-methanol-water (65:35:7 by volume) solution as the mobile phase. After chromatography, PAF and lyso-PAF were visualized by spraying with 5% ammonium molybdate sulfate and heating.

In the colorimetric assay, the reactions were initiated by mixing 100 μl of 20 mM Tris-HCl (pH 8.0), containing 2-thio PAF at various concentrations and 100 μl of 20 mM Tris-HCl containing 2.1 nM SsE^{M28} and 0.5 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) at room temperature in a 96-well plate. The absorbance at 414 nm of the reactions was recorded every 9 s using a SPECTRA^{Max} 384 Plus spectrophotometer (Molecular Devices, Sunnyvale, CA), and the time course of ΔA₄₁₄ was used to determine initial rates of hydrolysis of 2-thio PAF. For detecting PAF-AH activity in the supernatant of GAS cultures, 100 μl of culture supernatant from the mid-exponential growth phase (OD₆₀₀ = 0.35) was mixed with 40 μl of a solution containing 0.88 mM 2-thio PAF and 1.26 mM DTNB.

The standard assay was performed as described previously (36). Briefly, 0.1 mM PAF solution was prepared by mixing 42 μl of unlabeled PAF and 50 μl of [acetyl-³H]PAF in 4 ml of phosphate-buffered saline (PBS; pH 7.2). In this solution, the final molar ratio of unlabeled and labeled PAF was determined to be 2,547:1. Then, 10 μl of testing protein in PBS (pH 7.2) was mixed with 40 μl of PAF at various concentrations and incubated at 37°C for 30 min. After incubation, 50 μl of 0.1 M acetic acid was added into the mixture, immediately followed by adding 1.5 ml of 0.1 M sodium acetate solution. The whole reaction mixture was then loaded onto a preconditioned octadecyl silica gel cartridge (catalog no. JTB 7020-03 from J.T. Baker), and the filtrate containing [³H]acetate was collected. After mixed with 4 ml of ScintiVerse BD LSC Cocktail (Fisher Scientific, Pittsburg, PA), [³H]radioactivity in the filtrate was determined using a Beckman LS 6500 Scintillation Counter. Mole numbers of hydrolyzed PAF were determined by measuring the [³H]acetate radioactivity in each reaction filtrate, which was calculated based on a standard curve and the specific radioactivity of the PAF solution.

Assay for esterase activity. The esterase activity of SsE was determined with potentiometric titration using a pH-stat (model 360; Denver Instruments, USA), as described previously (35). SsE was stirred together with each substrate at the specified concentrations at 25°C in 25 ml of 2.0 mM Tris-HCl (pH 7.6), and the released acid was titrated in real time with 0.02 M NaOH to keep pH at 7.6 for 3 min. Hydrolysis rate was measured from the slope of the plot of the volume of NaOH added versus time.

Inactivation of SsE^{M1} and SsE^{M28} by DFP. SsE^{M1} (0.4 mg/ml) or SsE^{M28} (1.2 mg/ml) in 100 μl of 20 mM Tris-HCl (pH 7.4) was incubated with DFP, a serine esterase-specific inhibitor (37), at concentrations from 0 to 100 μM at 37°C for 30 min, and the remaining activity of the treated enzyme was determined by using 100 mM vinyl propionate as described above in the assay for esterase activity.

Generation of MGAS6180 mutants. An *sse*^{M28} fragment encoding amino acids 63 to 255 of MGAS6180 was deleted using pGRVΔ*sse*^{M28} as previously described (17), yielding Δ*sse*^{MGAS6180}. pGRVΔ*sse*^{M28} was constructed as follows. The 3' and 5' flanking fragments of the target fragment were amplified from the MGAS6180 chromosomal DNA using two pairs of primers 5'-GGATCCTTGATAAGTAACCGTGAGCTT-3' (primer 1)/5'-CCCATCGATTCAACTTCAACAGTTGACATATTGATCAAAGTTAG-3' (primer 2) and 5'-TGTGTAAGTTGAATCGATGGGGGAGATGTATCAGGGCTAG-3' (primer 3)/5'-GGATCCTAAAGAAGTCGGTAATGCTCA-3' (primer 4), respectively. The two PCR fragments were fused together by subsequent overlap PCR via the underlined 21-bp complementary tag sequences in primers 2 and 3 using the primers 1 and

4. The fused PCR product was subcloned into pGRV (38) at the BamHI site.

A *covS* gene fragment encoding amino acids 39 to 468 of CovS in MGAS6180 and *Δsse^{MGAS6180}* was deleted using pGRV Δ *covS*^{M28} as described previously (17), yielding Δ *covS* and *Δsse Δ covS*. pGRV Δ *covS*^{M28} was constructed as follows. The 3' and 5' ~1,000-bp flanking fragments of the *covS* fragment were amplified from the MGAS6180 chromosomal DNA using the pairs of primers 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTGAAATAGTCTAGGATATGAG-3' (primer 5)/5'-ATGCTCTTGACATCAGTAGCAAGGCAATCAGTGTAAGGCAGA-3' (primer 6) and 5'-TTGCTACTGATGTCAAGACATGTAGATGGGTATCATTTACAG-3' (primer 7)/5'-GGGGACCACTTTGTACAAGAAAGCTGGGTAGTACCGAATCAAGAGTTA-3' (primer 8), respectively. The two PCR fragments were fused together by subsequent overlay PCR via the 21-bp complementary tag sequences that are underlined in primers 6 and 7 using the primers 5 and 8. The fused PCR product was first cloned into the donor vector pDONR221 in the BP reaction and then into pGRV-RFA in the LR reaction using the Gateway Cloning Technology. pGRV-RFA was modified from pGRV by cloning the reading frame cassette A of the Gateway Cloning system at the SmaI site.

Quantitative RT-PCR analysis. GAS strains were grown in THY to an OD₆₀₀ of 0.3. GAS bacteria were harvested by centrifugation and incubated at room temperature for 5 min after the addition of 2 volumes of RNeasy Protect (Qiagen, Valencia, CA) to ensure RNA integrity. Total RNA was isolated using RNeasy minikit (Qiagen). RNA preparations were verified to be free of DNA by PCR. The relative levels of specific gene transcripts were measured by one-step SYBR quantitative reverse transcription-PCR (RT-PCR) assay. Changes in the levels of gene expression were compared using the $\Delta\Delta C_T$ method with normalization to the control gene *gyrA*. All RNA samples were assayed in triplicate, and the data represent mean values $\pm$ the standard deviations (SD).

In trans overexpression of SsE^{M28}. The *sse^{M28}* gene of MGAS6180 was PCR cloned into pDCBB-FRA with the Gateway Technology according to the manufacturer's manual. pDCBB (39) was modified into pDCBB-RFA by inserting the blunt-ended reading frame cassette A into pDCBB at the EcoRV site. The *sse^{M28}* gene was PCR amplified using Phusion DNA polymerase (New England BioLabs, Ipswich, MA) and the primers 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTAATGATAATAATAAGGAGACTC-3' and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCAAC TGTACGTGAGCTTGA-3'. The underlined sequences were *attB* sequences for the BP clonase reaction. The PCR product was cloned into the donor vector pDONR221 using the BP clonase, yielding pDONR221-*sse^{M28}*. The *sse^{M28}* gene in pDONR221 was transferred into pDCBB-RFA by the LR clonase, yielding pDCBB-*sse^{M28}*. The cloned *sse^{M28}* gene was confirmed by PCR and DNA sequencing. pDCBB-*sse^{M28}* or pDCBB (vector control) was introduced into MGAS6180 and its Δ *sse Δ covS* mutant via electroporation, as described previously (38).

In trans expression of *sse^{M28}* in MGAS6180 and the Δ *sse Δ covS* mutant was evaluated by Western blotting with mouse anti-SsE^{M28} antisera. MGAS6180 or the Δ *sse Δ covS* mutant containing pDCBB-*sse^{M28}* or pDCBB (vector control) was grown in protein-reduced Todd-Hewitt broth supplemented with 0.2% yeast extract (THY) (PR-THY) in the presence of 10 μ g of chloramphenicol/ml to an OD₆₀₀ of 0.35, and the cultures were centrifuged to obtain supernatant. Proteins in 10 ml of supernatant were precipitated with 30 ml of cold ethanol, and the precipitates were pelleted by centrifugation and dissolved in 200 μ l of 1 $\times$ sodium dodecyl sulfate (SDS) loading buffer. PR-THY was prepared by passing THY through a membrane filter with a 10,000-molecular-weight cutoff. Western blotting was performed as described previously (33). Proteins in 10 μ l of the obtained samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes (BioTrace NT; Pall Corp.) with Towbin transfer buffer using a Trans-Blot SD semidry transfer cell (Bio-Rad) at 15 V for 45 min. The membrane was blocked with 1:20 Amersham Liquid Block in Tween buffer (0.1% Tween 20 in PBS) for 1 h and subsequently incubated for 1 h with mouse anti-SsE^{M28} or anti-Spy0019 (control) antiserum added to

the block solution at 1:500 dilution. The membrane was then rinsed twice and washed for 15 min for three times with 0.1% Tween 20 in PBS. The membrane was incubated for 1 h with 1:2,000 goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) in the block solution and washed as described above. Antibody-antigen interaction was visualized by enhanced chemiluminescence.

Mouse infections. All animal experimental procedures were carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. The protocol for the animal procedures was approved by the Institutional Animal Care and Use Committee at Montana State University (permit 2011-57).

To prepare anti-SsE^{M28} antisera, 2 female BALB/c mice (5 weeks old) obtained from Charles River Laboratories were immunized subcutaneously on day 1 and then boosted with 50 μ g of purified recombinant SsE^{M28} suspended in 50 μ l of aluminum hydroxide gel (Sigma) on day 14. Immune antiserum was collected 2 weeks after the boost.

For subcutaneous infection of mice, GAS without or with plasmid was grown to the mid-exponential phase in THY without and with 10 mg of chloramphenicol/liter, respectively. The bacteria were harvested by centrifugation and then first washed three times with and then resuspended in pyrogen-free Dulbecco phosphate-buffered saline (DPBS) in the desired doses. Groups of female CD1 mice (5 weeks old; Charles River Laboratory) were subcutaneously infected with 0.2 ml of GAS suspension in DPBS at OD₆₀₀ of 3.0, and inocula were determined by plating. Mice were sacrificed at 24 h after inoculation to collect skin for measurement of the infected lesion size and neutrophil infiltration. The 24-h time point was chosen based on pilot test results that neutrophil levels and skin lesion sizes did not change much after 24 h after GAS inoculation.

Quantification of neutrophil infiltration. The skin around infection site was peeled off, and infection area in the skin was recognized by the boundary of inflammation in its picture and measured by using the area measurement tool of the Adobe Acrobat 9 software program (Adobe Systems, Inc.). The skin containing the infection area was excised for neutrophil measurement. Numbers of recruited neutrophils in the infected skin samples were determined by the myeloperoxidase assay, as described previously (8, 40). The skin samples were grinded in 0.5% hexadecyltrimethylammonium bromide in 50 mM potassium and sonicated on ice for 15 s to extract myeloperoxidase. The samples were frozen and thawed for three times, sonicated, and centrifuged at 16,000 $\times$ g for 5 min. The myeloperoxidase activity in the supernatant obtained was measured in 0.2 ml of 50 mM phosphate buffer (pH 6.0), containing the supernatant, 0.167 mg of *o*-dianisidine dihydrochloride/ml, and 0.001% hydrogen peroxide. The change in absorbance at 460 nm (ΔA_{460}) was recorded with time with a SPECTRA^{Max} 384 Plus spectrophotometer (Molecular Devices, Sunnyvale, CA). The myeloperoxidase activity, $\Delta A_{460}/\text{min}$, was converted into the number of neutrophils using a standard curve of myeloperoxidase activities using known numbers of murine neutrophils.

Statistical analyses. Statistical analyses of the data of the animal experiments were performed using the GraphPad Prism software with the two-tailed, unpaired Student *t* test with Welch's correction and the analysis of variance Tukey's multiple-comparison test.

RESULTS

PAF-AH activity of SsE^{M28}. Recombinant SsE^{M28} was purified to apparent homogeneity (Fig. 1A). Whether SsE^{M28} hydrolyzes PAF into lyso-PAF was determined by thin-layer chromatography (TLC) analysis. PAF C16 (0.29 mM) was incubated with 7 μ M SsE^{M28} for 20 min, and the reaction was stopped by the addition of 50% acetonitrile. SsE^{M28}-treated PAF migrated the same distance in TLC as lyso-PAF, indicating that PAF was converted into lyso-PAF in the treatment with SsE^{M28} (Fig. 1B). To confirm that the PAF hydrolysis was due to the enzymatic activity of SsE^{M28}, we performed a control experiment using PAF without addition of SsE^{M28}. PAF from this sham treatment retained the migration rate

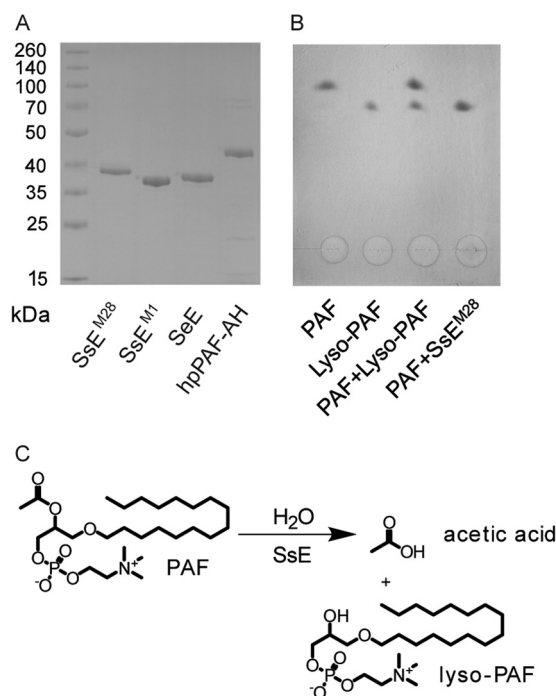


FIG 1 SsE^{M28}-catalyzed hydrolysis of PAF. (A) SDS-PAGE analysis of recombinant SsE^{M28}, SsE^{M1}, SsE, and hpPAF-AH. (B) TLC analysis of SsE^{M28}-catalyzed conversion of PAF into lyso-PAF. Samples from left to right show sham-treated PAF, authentic lyso-PAF, a mixture of authentic PAF and lyso-PAF, and SsE^{M28}-treated PAF. (C) Chemical reaction scheme for SsE-catalyzed hydrolysis of PAF.

of PAF in TLC (Fig. 1B), indicating no hydrolysis of PAF in the absence of SsE^{M28}. These results indicate that SsE^{M28} can hydrolyze the acetyl ester bond in PAF and thus has the PAF-AH activity (Fig. 1C).

Enzymatic parameters of SsE^{M28}, SsE^{M1}, and hpPAF-AH for PAF hydrolysis. The Michaelis constant (K_m) is associated with the affinity of an enzyme for substrate, and the catalytic rate constant (k_{cat}) is a measure of the catalytic production of product at saturated substrate concentrations for the enzyme. These parameters provide information on whether an enzyme efficiently targets a substrate. To determine whether SsE^{M28} and SsE^{M1} target PAF, we compared the K_m and k_{cat} of SsE^{M28} and SsE^{M1} in PAF hydrolysis with an authentic PAF-AH, hpPAF-AH. In the colorimetric assay, PAF-AH catalyzes the hydrolysis of the acetyl thio-ester bond of 2-thio-PAF, a homologue of PAF, yielding a free thiol, which reacts with DTNB to form 2-nitro-5-thiobenzoate with an absorbance peak at 414 nm (A_{414}). The time courses of ΔA_{414} in Fig. 2A show that the hydrolysis of 2-thio-PAF was very fast even when SsE^{M28} concentration was at 1.5 nM. The apparent K_m of the reaction was small, and low PAF concentrations had to be used to obtain the enzymatic parameters. The ΔA_{414} time course in the first 36 s of the reactions (Fig. 2B) was used to calculate initial reaction rates at different 2-thio-PAF concentrations. Because significant portions of 2-thio-PAF had been hydrolyzed at time zero when the measurement started, 2-thio-PAF concentrations at time zero was adjusted by subtracting the hydrolyzed portion, as described previously (8). The relationship of the initial rates versus the adjusted [2-thio-PAF] fits the Michaelis-Menten equation (Fig. 2C). The observed rates as a function of [2-thio-

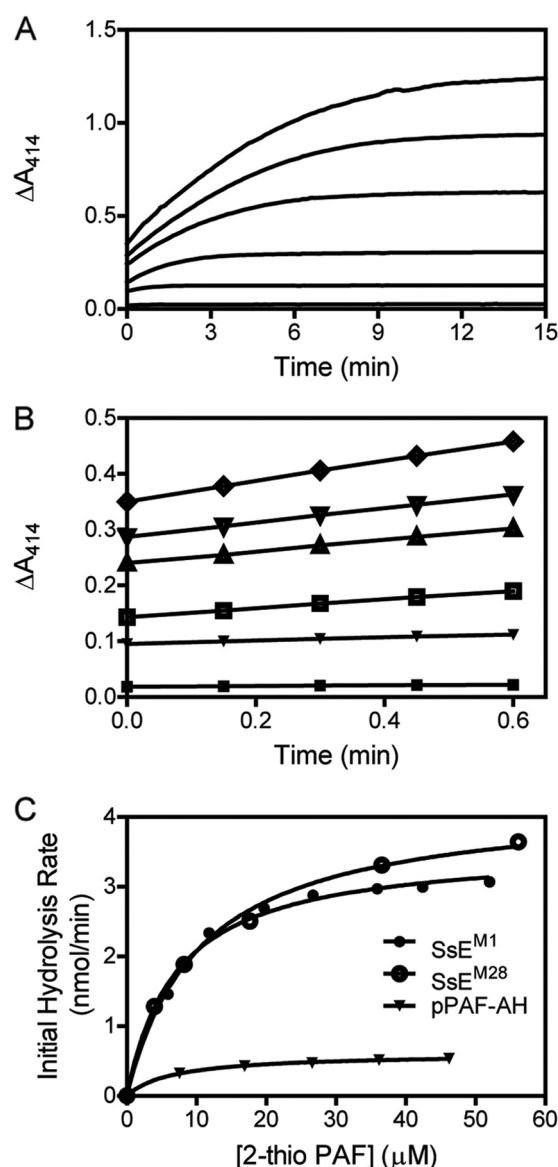


FIG 2 PAF-AH activity of SsE^{M28}, SsE^{M1}, and hpPAF-AH assessed using 2-thio-PAF. (A) Time courses of absorbance change at 414 nm (A_{414}) after mixing 2.1 nM SsE^{M28} with 2-thio PAF at 5, 10, 20, 40, 60, and 80 μ M, which correspond to the curves from bottom to top. (B) Linear regression of A_{414} change within 36 s in panel A to obtain initial rates of 2-thio PAF hydrolysis. (C) Initial rates of SsE^{M28}, SsE^{M1}, and hpPAF-AH-catalyzed hydrolysis of 2-thio PAF as a function of 2-thio PAF concentration.

PAF] also fit the Michaelis-Menten equation in the SsE^{M1}- and human pPAF-AH-catalyzed reactions (Fig. 2C). All the proteins had similar apparent K_m (average $\pm$ the SD: $10.4 \pm 1.2 \mu$ M for SsE^{M28}, $7.1 \pm 0.9 \mu$ M for SsE^{M1}, and $6.7 \pm 0.5 \mu$ M for hpPAF-AH) (Table 1); however, the k_{cat} values of SsE^{M28} (average $\pm$ the SD: $64.3 \pm 2.3 \text{ s}^{-1}$) and SsE^{M1} ($69.6 \pm 2.1 \text{ s}^{-1}$) were >4 -fold greater than that of hpPAF-AH ($15.4 \pm 0.2 \text{ s}^{-1}$).

To further examine the PAF-AH activity of SsE^{M28}, a mixture of [acetyl-³H]PAF and cold PAF was incubated with SsE^{M28}, and an aliquot was taken from the reaction solution to measure the radioactivity of [³H]acetate that was released in the PAF hydrolysis. Levels of [³H]acetate increased linearly with time (Fig. 3A).

TABLE 1 $V_{\max,app}$ and $K_{m,app}$ of PAF acetylhydrolases

Enzyme (kDa)	Mean $\pm$ SD			
	2-Thio-PAF		[acetyl- ^{3} H]PAF	
	$V_{\max,app}$ (s^{-1})	$K_{m,app}$ (μ M)	$V_{\max,app}$ (s^{-1})	$K_{m,app}$ (μ M)
SsE ^{M1} (35.8)	69.6 $\pm$ 2.1	7.1 $\pm$ 0.9	373 $\pm$ 26	24.9 $\pm$ 4.5
SsE ^{M28} (35.7)	64.3 $\pm$ 2.3	10.4 $\pm$ 1.2	467 $\pm$ 68	182.9 $\pm$ 38.9
Human pPAF-AH (46.4)	15.4 $\pm$ 0.2	6.7 $\pm$ 0.5	12 $\pm$ 1.0	6.8 $\pm$ 1.9
SeE (36.9)	18.7 $\pm$ 2.7	8.0 $\pm$ 3.5		

The release of [^{3} H]acetate was dose dependent on [SsE^{M28}] (Fig. 3B). These results confirm the PAF-AH activity of SsE^{M28}. The procedure of the standard PAF-AH assay (36) was then followed to compare the PAF-AH activity of SsE^{M28}, SsE^{M1}, and hpPAF-AH using [acetyl- ^{3} H]PAF. [Acetyl- ^{3} H]PAF at different concentrations was incubated with each PAF-AH for 30 min, and [^{3} H]acetate from the reactions was quantified to calculate the rate of the PAF hydrolysis reactions. The plots of the observed rates versus [PAF] in all the three enzymatic reactions fit the Michaelis-Menten equation (Fig. 3C), resulting in K_m and k_{cat} for each of the enzymes (Table 1). SsE^{M28} had a k_{cat} (average $\pm$ the SD: 466.5 $\pm$ 67.7 s^{-1}) that was slightly higher than that of SsE^{M1} (372.9 $\pm$ 25.5 s^{-1}) and was 37.6 times that of hpPAF-AH (12.4 $\pm$ 0.8 s^{-1}). However, the K_m value of SsE^{M28} (182.9 $\pm$ 38.9 μ M) was 7.3 and 26.9 times that of SsE^{M1} (24.9 $\pm$ 4.5 μ M) and hpPAF-AH (6.8 $\pm$ 1.9 μ M), respectively. Both the SsE^{M28} and the SsE^{M1} proteins were apparently pure, and the purity of hpPAF-AH was >90% (Fig. 1A). Thus, these K_m and k_{cat} values of the proteins in catalyzing the hydrolysis of 2-thio-PAF and PAF indicate that SsE^{M28} and SsE^{M1} have high affinities for PAF and are more potent PAF-AH than hpPAF-AH.

SsE^{M28} appears to be the only PAF-AH produced by MGAS6180. PAF-AHs are members of the phospholipase A₂ family. MGAS6180 also produces another phospholipase A₂ protein, SlaA (41, 42). To determine whether SlaA also has the PAF-AH activity, we tested hydrolysis of 2-thio-PAF by SlaA. The activity was barely detectable when 3.1 μ M SlaA was used, and the specific activity of SlaA for 2-thio-PAF hydrolysis was just 0.003 μ mol/min/mg for Sla, which was 4 orders of magnitude lower than that of SsE^{M1}. Thus, SlaA does not have the PAF-AH activity. Consistent with this conclusion, deletion of the *sse* gene in MGAS6180 abolished the PAF-AH activity in the culture supernatant of MGAS6180 (see Fig. 6B). It appears that SsE^{M28} is the only PAF-AH produced by MGAS6180.

S. equi SeE protein has the PAF-AH activity. The SeE protein of *S. equi* shares 61% identity in amino acid sequence with SsE^{M1} (35) and may have PAF-AH activity. Indeed, SeE efficiently converted PAF into lyso-PAF according to TLC analysis (data not shown). We also determined the k_{cat} and K_m of SeE for the hydrolysis of 2-thio-PAF to be 18.7 $\pm$ 2.7 s^{-1} and 8.0 $\pm$ 3.5 μ M, respectively. These measurements indicate that SeE is also a potent PAF-AH.

SsE^{M1} and SsE^{M28} are serine esterases. Both SsE^{M1} and SsE^{M28} have the GXSG motif that is present in carboxylesterases, suggest that they are serine carboxylhydrolases. To confirm this suggestion, we tested whether DFP inactivates SsE^{M1} and SsE^{M28}. The enzymatic activity of SsE^{M1} and SsE^{M28} was inactivated in a dose-

dependent manner by DFP (Fig. 4). Thus, SsE^{M1} and SsE^{M28} use a serine residue for its catalysis. This result is consistent with the finding that the replacement of the Ser¹⁷⁸ residue in SsE^{M1} with Ala abolishes the enzymatic activity of SsE^{M1} (32).

Substrate specificity of SsE^{M1} and SsE^{M28}. Besides PAF, hpPAF-AH can hydrolyze a variety of esters (28). To test whether SsE^{M1} and SsE^{M28} are specific PAF-AHs, we examined the sub-

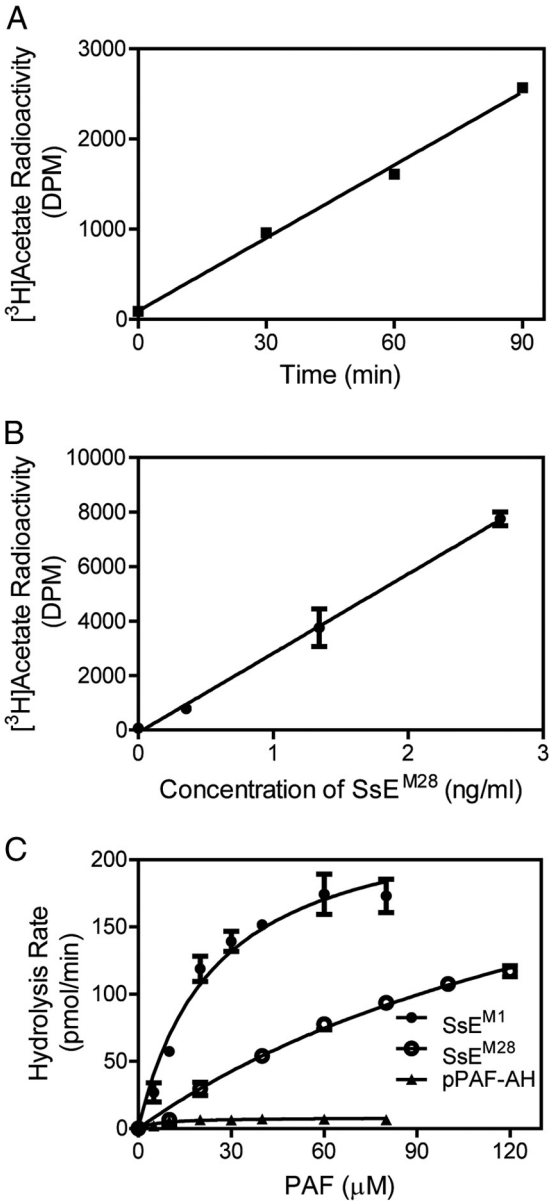


FIG 3 PAF-AH activity of SsE^{M28}, SsE^{M1}, and hpPAF-AH assessed using [acetyl- ^{3} H]PAF. (A) Time course of released [^{3} H]acetate radioactivity in a reaction containing 0.023 ng of SsE^{M28} and 40 μ M PAF. (B) Dose dependence of released [^{3} H]acetate radioactivity on SsE^{M28} concentration in SsE^{M28}-catalyzed hydrolysis of PAF. (C) The rates of SsE^{M28}-, SsE^{M1}-, and hpPAF-AH-catalyzed hydrolysis of PAF as a function of PAF concentration. The rates were calculated from the released [^{3} H]acetate after a 30-min reaction time. The concentrations of SsE^{M28}, SsE^{M1}, and hpPAF-AH used in the assay were 37.4, 14.5, and 215.5 pM, respectively. The presented data are adjusted from the observed data for a concentration of 215.5 pM for all of the proteins for comparison. Data are from a representative duplicate experiment.

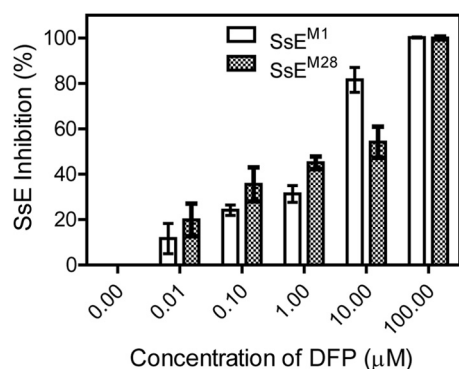


FIG 4 Inactivation of SsE^{M28} and SsE^{M1} by DFP. Each SsE protein was incubated with DFP at 37°C for 30 min, and the remaining esterase activity of the treated SsE was determined using 100 mM vinyl propionate. Presented are the percentages of inactivation at the indicated DFP concentrations.

strate specificity of the SsE proteins by testing various esters using potentiometric titration with a pH-stat in which NaOH was automatically pumped in to neutralize released acid and keep pH of the reaction solution constant. SsE^{M1}-catalyzed hydrolysis of ethyl acetate is used to show a typical titration curve. After SsE^{M1} was mixed with 50 mM ethyl acetate, the accumulative NaOH volume pumped increased linearly with time (Fig. 5A), giving a slope of 0.073 ml of 0.02 M NaOH min⁻¹, which was used to calculate the hydrolysis rate. The hydrolysis rate depended hyperbolically on the substrate concentration and fit the Michaelis-Menten equation (Fig. 5B). No hydrolysis was observed in a control reaction without SsE^{M1} (Fig. 5A).

We tested a series of triglycerides, including triacetyl glycerol, tripropionyl glycerol, tributiroyl glycerol, and trioctanoyl glycerol. One feature in this test is that both SsE^{M1} and SsE^{M28}, like hpPAF-AH, had the highest activity against triacetyl glycerol (Table 2). Because the solubility of these triglycerides in water decreases with the increase in the chain length of the acyl group, it is possible that the activity-decreasing trend with the acyl chain length-increasing triglycerides is determined by their solubility. We examined this possibility by testing the hydrolysis of vinyl acetate, vinyl propionate, and vinyl butyrate. These compounds all have good solubility, and their solubility should not be a factor. The specific activity of both SsE^{M1} and SsE^{M28} still dramatically decreased with the increase in the acyl chain length of this series of esters, supporting the notion that both SsE proteins have genuine, optimal activities against acetyl esters.

We then tested whether the alcohol moiety of acetyl esters contributes to the substrate specificity of both SsE proteins. Both SsE^{M1} and SsE^{M28} had lower specificity activity against ethyl acetate than triacetyl glycerol and had no activity against acetylcholine. Thus, the alcohol moiety of acetyl esters plays a role in the substrate activity. It is noticeable that SsE^{M28} had lower activities against triglycerides and other nonlipophilic esters than SsE^{M1} (Table 2).

Effects of *sse*^{M28} deletion on neutrophil recruitment and skin invasion. As an important lipid mediator, PAF can activate platelets and neutrophils and is involved in inflammation. Since SsE^{M28} has the PAF-AH activity, we hypothesize that SsE^{M28} can detrimentally affect neutrophil responses to GAS infection, contributing to GAS pathogenesis. The effect of SsE^{M1} on neutrophil recruitment and skin invasion is more profound in M1 strains that

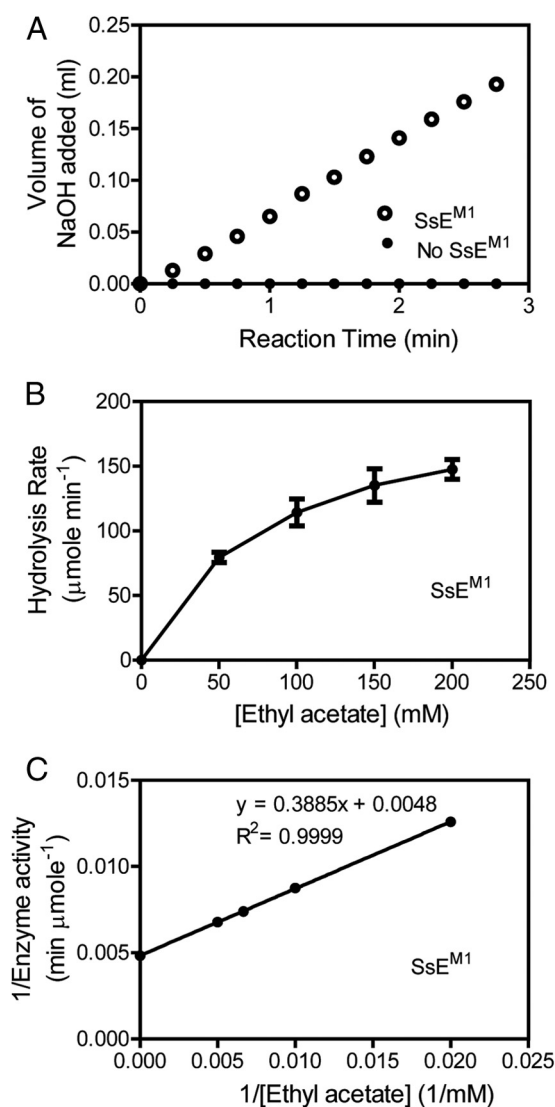


FIG 5 SsE^{M1}-catalyzed hydrolysis of ethyl acetate in potentiometric titration. (A) Accumulative volume of 0.02 M NaOH titrated as a function of time during a reaction of 50 mM ethyl acetate in the absence (●) or presence (○) of 0.5 μM SsE^{M1}. (B) Hydrolysis rates calculated from the slope in panel A at various ethyl acetate concentrations. (C) Double-reciprocal analysis of enzyme activity calculated from the slope in panel B at various ethyl acetate concentrations.

carry a null *covS* mutation than strains carrying wild-type *CovS* because SsE^{M1} is expressed at higher levels in isolates with null *covS* mutations (8, 17, 33). Thus, we deleted the *sse* gene in MGAS6180 and the *covS* gene in both MGAS6180 and its Δsse mutant for examining the effect of *sse*^{M28} deletion on infection outcomes. As expected, deletion of *covS* in MGAS6180 did not change the levels of the *emm* transcript for the M protein but enhanced the transcript levels of *hasA* and *sse* (Fig. 6A), which are the known targets of *CovRS*. The *sse* and *covS* deletions in MGAS6180 abolished and increased the PAF-AH activity in the culture supernatant, respectively (Fig. 6B). It should be noted that the *covS* deletion-caused 5.6-fold change in the levels of *sse* transcript in MGAS6180 (Fig. 6A) was smaller than the *covS* deletion-caused 10-fold increase in the levels of *sse* transcript in MGAS2221

TABLE 2 Substrate specificity of SsE^{M28}, SsE^{M1}, and pPAF-AH^a

Substrate	Mean ± SD					
	SsE ^{M28}		SsE ^{M1}		Human pPAF-AH ^b	
	<i>V</i> _{max,app} (s ⁻¹)	<i>K</i> _{m,app} (mM)	<i>V</i> _{max,app} (s ⁻¹)	<i>K</i> _{m,app} (mM)	<i>V</i> _{max,app} (s ⁻¹)	<i>K</i> _{m,app} (mM)
Triacetylgllycerol	24.1 ± 2.1	18.9 ± 3.4	270.0 ± 23.1	13.8 ± 2.7	33 ± 2	131 ± 19
Tripropionylglycerol	12.4 ± 1.9	9.3 ± 3.8	207.2 ± 19.2	6.3 ± 1.9		
Tributyrylglycerol	3.8 ± 0.7	3.1 ± 1.4	25.8 ± 3.1	2.3 ± 0.8	12 ± 1	0.136 ± 0.029
Trioctanoylglycerol	0		0		≤5	
Vinyl acetate	226.6 ± 24.2*		2,734.5 ± 45.2*			
Vinyl propionate	98.1 ± 4.5*		1,071.2 ± 40.1*			
Vinyl butyrate	6.5 ± 0.1*		118.0 ± 6.0*			
Ethyl acetate	14.0 ± 1.1	162.1 ± 25.9	123.8 ± 0.7	81.0 ± 1.2		
Acetylcholine	0		0			

^a *, Data were obtained at a 100 mM ester concentration.
^b Data were obtained from reference 28.

(33). This difference could be due to the following difference in *sse* expression between the two wild-type strains: the PAF-AH activity of SsE was detected in the culture supernatant of MGAS6180 but not MGAS2221 (data not shown). An additional difference between MGAS6180 and MGAS2221 was that the SpeB activity was detected in the supernatant of overnight MGAS2221 culture but not MGAS6180 (data not shown). It appears that there is a difference between MGAS6180 and MGAS2221 in CovRS-mediated regulation of *speB* and *sse*. However, CovRS still negatively regulates *sse* in MGAS6180, and deletion of *covS* in MGAS6180 enhances SsE production.

The Δsse mutant caused smaller lesions ($63.6 \pm 3.6 \text{ mm}^2$) than MGAS6180 ($72.3 \pm 6.8 \text{ mm}^2$), but the difference was not significant ($P = 0.5476$) (Fig. 6C) in subcutaneous infection of CD1 mice, and the levels of neutrophils at the parent and Δsse mutant sites were similar (Fig. 6D). However, deletion of *sse* in

MGAS6180 significantly reduces skin invasion and enhances neutrophil recruitment under the background of a null *covS* mutation. Compared to MGAS6180, the deletion of *covS* in MGAS6180 increased lesion sizes by 3.1-fold ($P < 0.0001$) (Fig. 6C) and reduced neutrophil recruitment by 68% ($P < 0.05$) (Fig. 6D). The *covS* deletion-caused increase in skin invasion and reduction in neutrophil recruitment were largely reversed by *sse* deletion. The $\Delta sse \Delta covS$ mutation caused a lesion size of $88.4 \pm 8.8 \text{ mm}^2$, which was 39% of that caused by the $\Delta covS$ mutation ($226.3 \pm 36.3 \text{ mm}^2$) ($P < 0.0001$) (Fig. 6C), and the levels of neutrophils at $\Delta sse \Delta covS$ sites [$(4.9 \pm 1.0) \times 10^5$ neutrophils/ mm^2] were 3.3 times that at $\Delta covS$ sites [$(1.5 \pm 0.4) \times 10^5$ neutrophils/ mm^2] ($P < 0.05$) (Fig. 6D). Thus, SsE^{M28} significantly contributes to skin invasion and inhibition of neutrophil recruitments under the nonfunctioning *covS* background.

To further confirm the contribution of the *sse* deletion to the

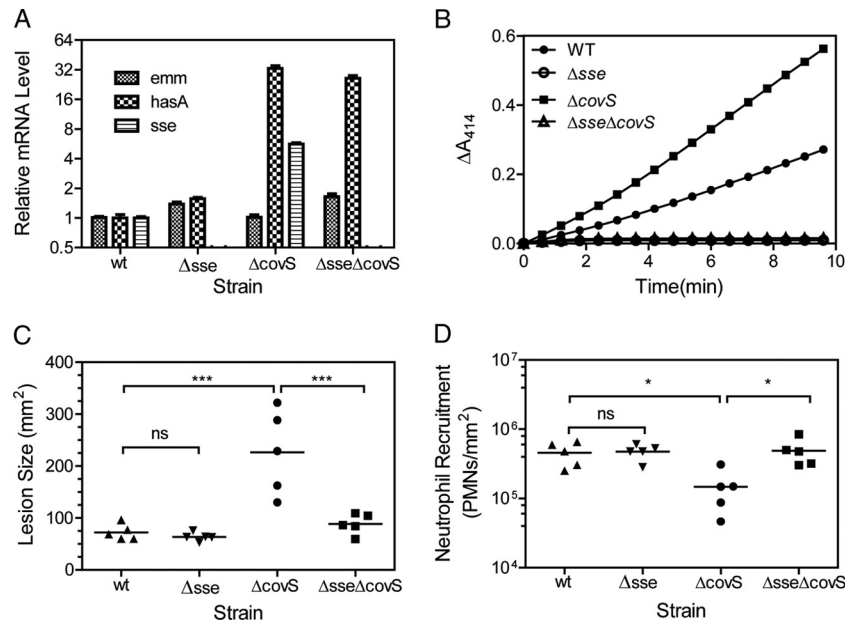


FIG 6 Effect of *sse*^{M28} deletion on GAS skin invasion and inhibition of neutrophil recruitment in subcutaneous infection of mice. (A) Relative levels of *emm*, *hasA*, and *sse* transcripts in MGAS6180 and its Δsse , $\Delta covS$, and $\Delta sse \Delta covS$ mutants at the mid-log growth phase. (B) PAF-AH activity in a 100- μ l culture supernatant of MGAS6180 and its mutants at the mid-log growth phase, which was monitored as the change in A_{414} in the 2-thio-PAF hydrolysis assay. (C and D) Lesion sizes (C) and neutrophil infiltration at lesion sites (D) in mice subcutaneously infected with 7.9×10^8 CFU of MGAS6180, 7.2×10^8 CFU of Δsse mutant, 9.0×10^8 CFU of $\Delta covS$ mutant, or 9.5×10^8 CFU of $\Delta sse \Delta covS$ mutant. Statistical analysis: *, **, or ***, significant; ns, not significant.

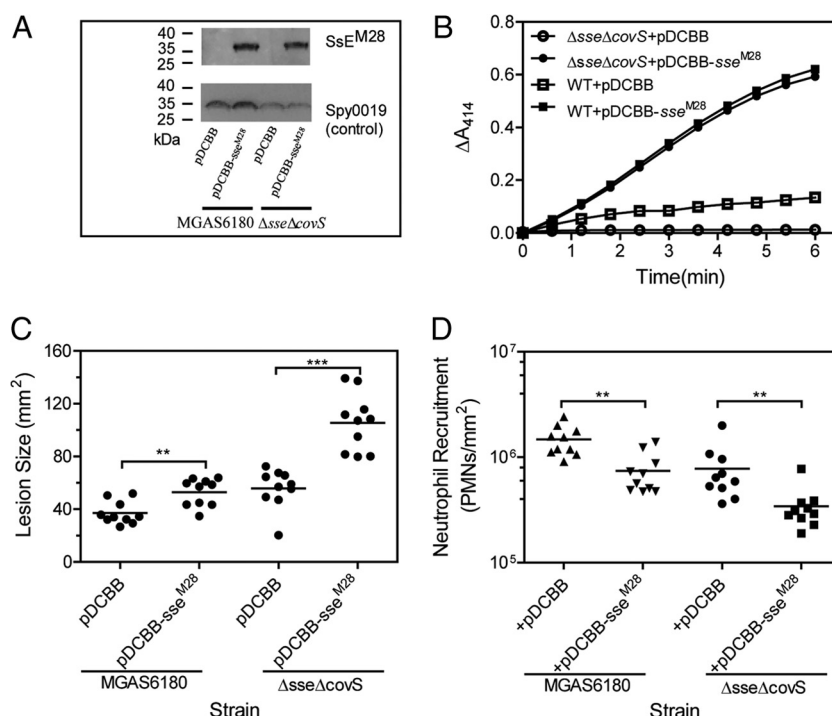


FIG 7 Effects of *in trans* overexpression of Sse^{M28} on skin invasion and neutrophil recruitment in subcutaneous GAS infections. (A and B) *In trans* expression of Sse^{M28} in MGAS6180/pDCBB-*sse*^{M28} and $\Delta sse \Delta covS$ strain/pDCBB-*sse*^{M28} detected by Western blotting (A) and the 2-thio-PAF hydrolysis assay (B). $\Delta sse \Delta covS$ strain/pDCBB was the vector control, and Spy0019 was a secreted protein control. (C and D) Lesion sizes (C) and neutrophil infiltration at lesion sites (D) in mice subcutaneously infected with 4.9×10^8 CFU of MGAS6180/pDCBB, 4.6×10^8 CFU of MGAS6180/pDCBB-*sse*^{M28}, 6.6×10^8 CFU of $\Delta sse \Delta covS$ strain/pDCBB, or 5.4×10^8 CFU of $\Delta sse \Delta covS$ strain/pDCBB-*sse*^{M28}. Statistical analysis: *, **, or ***, significant; ns, not significant.

inhibition of neutrophil recruitment and skin invasion, we complemented the *sse* deletion by *in trans* expression of Sse^{M28} in $\Delta sse \Delta covS$ mutants. Western blotting detected a strong Sse^{M28} band in the culture supernatant of $\Delta sse \Delta covS$ mutants carrying pDCBB-*sse*^{M28}, but no Sse^{M28} band was detected in the culture supernatant of $\Delta sse \Delta covS$ mutants carrying pDCBB (vector control), whereas similar levels of Spy0019, a secreted protein, were detected in the two samples (Fig. 7A). The culture supernatant of $\Delta sse \Delta covS$ mutant/pDCBB-*sse*^{M28} had high PAF-AH activity, whereas no PAF-AH activity was detected in the supernatant of $\Delta sse \Delta covS$ strain/pDCBB culture (Fig. 7B), confirming the Western blotting results. The lesion size in $\Delta sse \Delta covS$ strain/pDCBB-*sse*^{M28} infection was 105.6 ± 7.0 mm², which was nearly as twice as that in $\Delta sse \Delta covS$ strain/pDCBB infection (55.8 ± 4.7 mm²) ($P < 0.0001$) (Fig. 7C). The levels of neutrophils at the $\Delta sse \Delta covS$ strain/pDCBB-*sse*^{M28} site [$(3.4 \pm 0.5) \times 10^5$ neutrophils/mm²] was less than half of that at the $\Delta sse \Delta covS$ MGAS6180 strain/pDCBB site [$(7.7 \pm 1.5) \times 10^5$ neutrophils/mm²] ($P = 0.0011$) (Fig. 7D). Thus, Sse^{M28} has a similar function to reduce neutrophil infiltration and increase skin invasion and is subject to the similar CovRS regulation as Sse^{M1}.

Effect of *in trans* expression of *sse*^{M28} in MGAS6180 on neutrophil recruitment and skin invasion. The *sse*^{M28} deletion results described above raise a question on whether Sse^{M28} exerts an effect only when GAS lacks the functioning CovRS. To examine this issue, we examined the effect of *in trans* expression of Sse^{M28} in MGAS6180 on infection outcomes. A weak Sse^{M28} band could be detected if a more concentrated MGAS6180/pDCBB sample was analyzed (data not shown). Western blotting and PAF-AH

activity analyses indicate the achievement of Sse^{M28} overexpression in MGAS6180 using pDCBB-*sse*^{M28} (Fig. 7A and B). MGAS6180/pDCBB-*sse*^{M28} caused greater lesions (53.0 ± 3.3 mm²) than MGAS6180/pDCBB (vector control) (37.1 ± 2.1 mm²) ($P = 0.0029$) (Fig. 7C). The *in trans* expression of Sse^{M28} in MGAS6180 reduced neutrophil infiltration [$(7.4 \pm 1.0) \times 10^5$ neutrophils/mm²] by 50% compared to the control [$(1.5 \pm 0.1) \times 10^6$ neutrophils/mm²] ($P = 0.0015$) (Fig. 7D). Thus, Sse^{M28} can detrimentally affect neutrophil infiltration and enhance skin invasion under the functional CovRS background when it is expressed at sufficient levels.

DISCUSSION

We report the findings on Sse, a secreted protein that is a protective antigen and virulence factor and functions in part by hydrolyzing PAF. First, Sse^{M28}, which represents the second group of Sse variants, also has potent PAF-AH activity despite of 37% sequence variation from Sse^{M1}, which represents the first group of Sse variants. Second, both Sse^{M1} and Sse^{M28} show higher activity against PAF than hpPAF-AH but have different activities against lipophilic esters and other esters, indicating that Sse^{M28} is a more specific PAF-AH and that Sse^{M1} is more like hpPAF-AH with a broader substrate specificity. Third, Sse^{M28} contributes to GAS invasion of the skin and inhibition of neutrophil recruitment. These findings provide new information on the function and functional mechanism of Sse.

Our results suggest that Sse^{M1} and Sse^{M28} were evolved to target PAF. First, Sse^{M1} and Sse^{M28} have higher k_{cat} and lower K_m against triacetyl glycerol than ethyl acetate, suggesting that acetyl

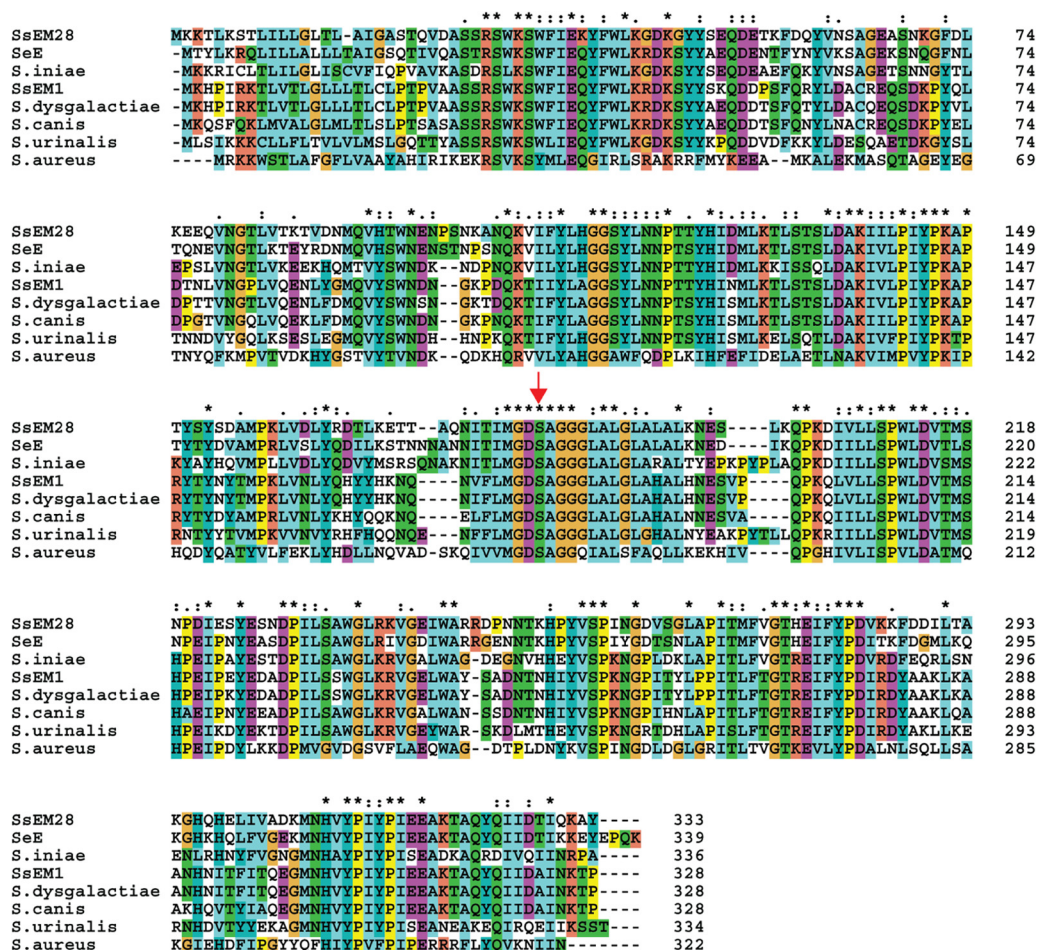


FIG 8 Amino acid alignment of SsE^{M1}, SsE^{M28}, and their homologues in six other bacterial pathogens. The conserved putative catalytic serine residue is indicated with the arrow. Selected SsE and SsE homologues are from MGAS5005 (GenBank access number YP_282770), MGAS6180 (YP_280915), *Streptococcus equi* subsp. *equi* (YP_002745890), *Streptococcus iniae* (ZP_11067786), *Streptococcus dysgalactiae* subsp. *equisimilis* (ZP_12400852), *Streptococcus canis* (ZP_10274453), *Streptococcus urinalis* (ZP_09136526), and *Staphylococcus aureus* subsp. *aureus* (ZP_04864376). The multiple alignments were performed by ClustalX 2.1.

attached to glycerol is more efficiently removed than acetyl attached to ethanol. These data support the targeting of SsE for PAF since PAF has its acetyl group attached to glycerol. Second and more importantly, both SsE^{M28} and SsE^{M1} have a k_{cat} value in the PAF hydrolysis that is 19- and 1.4-fold higher than in the triacetyl-glycerol hydrolysis, respectively, and, at the same time, their K_m values in the PAF hydrolysis are 103- and 554-fold lower than that in the triacetyl-glycerol hydrolysis, respectively. According to the Michaelis-Menten equation, these data clearly indicate that SsE^{M1} and SsE^{M28} have much higher affinity for PAF compared to triacetyl-glycerol and the other esters. Targeting PAF by SsE is further supported by the observations that the phospholipase A₂ Sla does not hydrolyze PAF and that SsE is the only PAF-AH detected in the MGAS6180 culture.

The analysis of the substrate specificity suggests that SsE^{M28} is a more specific PAF-AH compared to SsE^{M1} and hpPAF-AH and that SsE^{M1} and hpPAF-AH have similar broad substrate specificity. The substrate specificity of *S. equi* SeE (35) is similar to that of SsE^{M1}. SsE^{M28} possesses much lower activities against the triglycerides and the nonlipophilic esters than SsE^{M1} even though both the SsE proteins have similar activities against PAF and 2-thio-PAF. For example, the specific activities of SsE^{M1} against the three

short acyl chain triglycerides were 37 to 87 times greater than those of SsE^{M28}. Although SsE^{M1} has higher specific activity against PAF, 2-thio-PAF, and triglycerides than hpPAF-AH, the two proteins have similar relative activities against these esters. Compared to the other esters tested, SsE^{M28} has disproportionately higher activity against PAF and 2-thio-PAF among all the esters tested than SsE^{M1} and hpPAF-AH.

A high degree of variation in amino acid sequence between SsE^{M1} and SsE^{M28} would suggest that they might have evolved different functions. SsE^{M1} critically contributes to GAS skin invasion and inhibition of neutrophil recruitment, and this function of SsE^{M1} is at least partially mediated by its PAF-AH activity (8). SsE^{M28} also contributes to GAS skin invasion and inhibition of neutrophil recruitment. These results indicate that SsE^{M1} and SsE^{M28} have same function. SsE^{M1} and SsE^{M28} have similar PAF-AH activity. This preservation of the PAF-AH activity and function in SsE^{M1} and SsE^{M28} suggests that both of the proteins function through the PAF-AH activity.

Inhibition of neutrophil recruitment is one of the mechanisms for GAS to invade neutrophil responses. It has been known that GAS produces the peptidases ScpA and SpyCEP to degrade the C5a peptide and IL-8/CXC chemokines, respectively (3–7). SsE is

a recently found novel factor that contributes to GAS inhibition of neutrophil recruitment (8). All of these three factors are regulated by the two-component regulatory system CovRS (33, 43, 44). Hypervirulent GAS can almost completely inhibit neutrophil infiltration (4, 8, 45–47) and have enhanced expression of the *spyCEP* and *sse* genes (33, 43, 44). Null deletion and mutations of the *covS* gene cause the severe inhibition of neutrophil infiltration, and SsE is the most critical factor among SsE, ScpA, and SpyCEP for the *covS* deletion/mutation-mediated enhancement in innate immune evasion and virulence (17). The findings on SsE^{M28} suggest that the function of SsE in innate immune evasion by targeting PAF is conserved even the sequence of SsE^{M28} is extensively diverged from that of SsE^{M1}. GAS possesses either SsE^{M1}-type or SsE^{M28}-type SsE protein depending on the serotype. Apparently, GAS uses SsE to evade innate immune response regardless of the serotypes.

Like SsE^{M1}, SsE^{M28} also significantly contributes to skin invasion, which may be the consequence of the SsE-mediated reduction in the neutrophil responses or attributable to an unknown mechanism. In addition, both SsE^{M28} and SsE^{M1} (10, 19, 35) have a more substantial effect on neutrophil infiltration and skin infection for GAS strains with nonfunctional *covS* compared to strains with the wild-type *covS* gene. This is apparently due to higher expression levels of *sse* in null *covS* mutants of MGAS6180 and MIT1 GAS isolates.

The preservation of the PAF-AH activity of SsE^{M1}, SsE^{M28}, and their *S. equi* homologue SeE suggests that the function and functional mechanism of SsE is not limited to GAS. SsE^{M1} shares 33 to 94% sequence identity with a putative secreted protein in a number of bacterial pathogens, which notably include *Streptococcus dysgalactiae* subsp. *equisimili* (94% sequence identity), *Streptococcus dysgalactiae* subsp. *dysgalactiae* (86%), *Streptococcus canis* (82%), *Streptococcus urinalis* (64%), *Streptococcus equi* subsp. *zooepidemicus* (62%), *Streptococcus equi* subsp. *equi* (62%), *Streptococcus iniae* (61%), *Streptobacillus moniliformis* (41%), *Clostridium kluyveri* (38%), *Lactobacillus oris* (36%), *Lactobacillus antri* (36%), *Actinomyces coleocanis* (36%), and *Staphylococcus aureus* (33%). Sequence alignment of selected SsE homologues is presented in Fig. 8. Notably, *Streptococcus pneumoniae* does not have an SsE homologue; however, this pathogen produces the protein Pce that can hydrolyze the phosphocholine group of PAF (48). It is possible that a redundant SsE homologue targeting PAF is not needed because of the production of Pce.

ACKNOWLEDGMENTS

This study was supported in part by grants AI095704, AI097703, and GM103500-09 from the National Institutes of Health, USDA Animal Formula Fund, and the Montana State Agricultural Experimental Station.

REFERENCES

- Olsen RJ, Musser JM. 2010. Molecular pathogenesis of necrotizing fasciitis. *Annu. Rev. Pathol.* 5:1–31.
- O'Loughlin RE, Roberson A, Cieslak PR, Lynfield R, Gershman K, Craig A, Albanese BA, Farley MM, Barrett NL, Spina NL, Beall B, Harrison LH, Reingold A, Van Beneden C. 2007. The epidemiology of invasive group A streptococcal infection and potential vaccine implications: United States, 2000–2004. *Clin. Infect. Dis.* 45:853–862.
- Wexler DE, Chenoweth DE, Cleary PP. 1985. Mechanism of action of the group A streptococcal C5a inactivator. *Proc. Natl. Acad. Sci. U. S. A.* 82:8144–8148.
- Hidalgo-Grass C, Mishalian I, Dan-Goor M, Belotserkovsky I, Eran Y, Nizet V, Peled A, Hanski E. 2006. A streptococcal protease that degrades CXC chemokines and impairs bacterial clearance from infected tissues. *EMBO J.* 25:4628–4637.
- Edwards RJ, Taylor GW, Ferguson M, Murray S, Rendell N, Wrigley A, Bai Z, Boyle J, Finney SJ, Jones A, Russell HH, Turner C, Cohen J, Faulkner L, Sriskandan S. 2005. Specific C-terminal cleavage and inactivation of interleukin-8 by invasive disease isolates of *Streptococcus pyogenes*. *J. Infect. Dis.* 192:783–790.
- Zinkernagel AS, Timmer AM, Pence MA, Locke JB, Buchanan JT, Turner CE, Mishalian I, Sriskandan S, Hanski E, Nizet V. 2008. The IL-8 protease SpyCEP/ScpC of group A streptococcus promotes resistance to neutrophil killing. *Cell Host Microbe* 4:170–178.
- Sumby P, Zhang S, Whitney AR, Falugi F, Grandi G, Graviss EA, Deleo FR, Musser JM. 2008. A chemokine-degrading extracellular protease made by group A streptococcus alters pathogenesis by enhancing evasion of the innate immune response. *Infect. Immun.* 76:978–985.
- Liu M, Zhu H, Li J, Garcia CC, Feng W, Kirpotina LN, Hilmer J, Tavares LP, Layton AW, Quinn MT, Bothner B, Teixeira MM, Lei B. 2012. Group A streptococcus secreted esterase hydrolyzes platelet-activating factor to impede neutrophil recruitment and facilitate innate immune evasion. *PLoS Pathog.* 8:e1002624. doi:10.1371/journal.ppat.1002624.
- Perez-Casal J, Caparon MG, Scott JR. 1992. Introduction of the emm6 gene into an emm-deleted strain of *Streptococcus pyogenes* restores its ability to resist phagocytosis. *Res. Microbiol.* 143:549–558.
- Ashbaugh CD, Moser TJ, Shearer MH, White GL, Kennedy RC, Wessels MR. 2000. Bacterial determinants of persistent throat colonization and the associated immune response in a primate model of human group A streptococcal pharyngeal infection. *Cell. Microbiol.* 2:283–292.
- Lei B, DeLeo FR, Hoe NP, Graham MR, Mackie SM, Cole RL, Liu M, Hill HR, Low DE, Federle MJ, Scott JR, Musser JM. 2001. Evasion of human innate and acquired immunity by a bacterial homolog of CD11b that inhibits opsonophagocytosis. *Nat. Med.* 7:1298–1305.
- von Pawel-Rammingen U, Johansson BP, Björck L. 2002. IdeS, a novel streptococcal cysteine proteinase with unique specificity for immunoglobulin G. *EMBO J.* 21:1607–1615.
- Timmer AM, Timmer JC, Pence MA, Hsu LC, Ghochani M, Frey TG, Karin M, Salvesen GS, Nizet V. 2009. Streptolysin O promotes group A *Streptococcus* immune evasion by accelerated macrophage apoptosis. *J. Biol. Chem.* 284:862–871.
- 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.
- Heath A, DiRita VJ, Barg NL, Engleberg NC. 1999. A two-component regulatory system, CsrR-CsrS, represses expression of three *Streptococcus pyogenes* virulence factors, hyaluronic acid capsule, streptolysin S, and pyrogenic exotoxin B. *Infect. Immun.* 67:5298–5305.
- Federle MJ, McIver KS, Scott JR. 1999. A response regulator that represses transcription of several virulence operons in the group A *Streptococcus*. *J. Bacteriol.* 181:3649–3657.
- Li J, Zhu H, Feng W, Liu M, Song Y, Zhang X, Zhou Y, Bei W, Lei B. 2013. Regulation of inhibition of neutrophil infiltration by the two-component regulatory system CovRS in subcutaneous murine infection with group A *Streptococcus*. *Infect. Immun.* 81:974–983.
- Hanahan DJ, Demopoulos CA, Liehr J, Pinckard RN. 1980. Identification of platelet activating factor isolated from rabbit basophils as acetyl glyceryl ether phosphorylcholine. *J. Biol. Chem.* 255:5514–5516.
- Chao W, Olson MS. 1993. Platelet-activating factor: receptors and signal transduction. *Biochem. J.* 292:617–629.
- Venable ME, Zimmerman GA, McIntyre TM, Prescott SM. 1993. Platelet-activating factor: a phospholipid autacoid with diverse actions. *J. Lipid. Res.* 34:691–702.
- Benveniste J, Henson PM, Cochrane CG. 1972. Leukocyte-dependent histamine release from rabbit platelets: the role of IgE, basophils, and a platelet-activating factor. *J. Exp. Med.* 136:1356–1377.
- Shaw JO, Pinckard RN, Ferrigni KS, McManus LM, Hanahan DJ. 1981. Activation of human neutrophils with 1-O-hexadecyl-octadecyl-2-acetyl-sn-glycerol-3-phosphorylcholine (platelet activating factor). *J. Immunol.* 127:1250–1255.
- Tjoelker LW, Wilder C, Eberhardt C, Stafforini DM, Dietsch G, Schimpf B, Hooper S, Le Trong H, Cousens LS, Zimmerman GA, Yamada Y, McIntyre TM, Prescott SM, Gray PW. 1995. Anti-

- inflammatory properties of a platelet-activating factor acetylhydrolase. *Nature* 374:549–553.
24. Hattori K, Adachi H, Matsuzawa A, Yamamoto K, Tsujimoto M, Aoki J, Hattori M, Arai H, Inoue K. 1996. cDNA cloning and expression of intracellular platelet-activating factor (PAF) acetylhydrolase II: its homology with plasma PAF acetylhydrolase. *J. Biol. Chem.* 271:33032–33038.
 25. Hattori M, Arai H, Inoue K. 1993. Purification and characterization of bovine brain platelet-activating factor acetylhydrolase. *J. Biol. Chem.* 268: 18748–18753.
 26. Stafforini DM, Prescott SM, Zimmerman GA, McIntyre TM. 1996. Mammalian platelet-activating factor acetylhydrolases. *Biochim. Biophys. Acta* 1301:161–173.
 27. Stafforini DM, McIntyre TM, Zimmerman GA, Prescott SM. 1997. Platelet-activating factor acetylhydrolases. *J. Biol. Chem.* 272:17895–17898.
 28. Min JH, Wilder C, Aoki J, Arai H, Inoue K, Paul L, Gelb MH. 2001. Platelet-activating factor acetylhydrolases: broad substrate specificity and lipoprotein binding does not modulate the catalytic properties of the plasma enzyme. *Biochemistry* 40:4539–4549.
 29. Foulks JM, Weyrich AS, Zimmerman GA, McIntyre TM. 2008. A yeast PAF acetylhydrolase ortholog suppresses oxidative death. *Free Radic. Biol. Med.* 45:434–442.
 30. Yang J, Zhang Y, Xu J, Geng Y, Chen X, Yang H, Wang S, Wang H, Jiang X, Guo X, Zhao G. 2009. Serum activity of platelet-activating factor acetylhydrolase is a potential clinical marker for leptospirosis pulmonary hemorrhage. *PLoS One* 4:e4181. doi:10.1371/journal.pone.0004181.
 31. Navarro-Fernández J, Nechitaylo TY, Guerrero JA, Golyshina OV, García-Carmona F, Sánchez-Ferrer A, Golyshin PN. 2011. A novel platelet-activating factor acetylhydrolase discovered in a metagenome from the earthworm-associated microbial community. *Environ. Microbiol.* 13:3036–3046.
 32. Liu M, Zhu H, Zhang J, Lei B. 2007. Active and passive immunizations with the streptococcal esterase Sse protect mice against subcutaneous infection with group A streptococci. *Infect. Immun.* 75:3651–3657.
 33. Zhu H, Liu M, Sumby P, Lei B. 2009. The secreted esterase of group A streptococcus is important for invasive skin infection and dissemination in mice. *Infect. Immun.* 77:5225–5232.
 34. Lei B, Liu M, Voyich JM, Prater CI, Kala SV, DeLeo FR, Musser JM. 2003. Identification and characterization of HtsA, a second heme-binding protein made by *Streptococcus pyogenes*. *Infect. Immun.* 71:5962–5969.
 35. Xie G, Liu M, Zhu H, Lei B. 2008. Esterase SeE of *Streptococcus equi* ssp. *equi* is a novel nonspecific carboxylic ester hydrolase. *FEMS Microbiol. Lett.* 289:181–186.
 36. Stafforini DM, McIntyre TM. 2013. Determination of phospholipase activity of PAF acetylhydrolase. *Free Radic. Biol. Med.* 59:100–107.
 37. Whitaker JR. 1972. Principles of enzymology for the food sciences, p 481–501. Marcel Dekker, Inc, New York, NY.
 38. Liu M, Hanks TS, Zhang J, McClure MJ, Siemsen DW, Elser JL, Quinn MT, Lei B. 2006. Defects in ex vivo and in vivo growth and sensitivity to osmotic stress of group A *Streptococcus* caused by interruption of response regulator gene *vicR*. *Microbiology* 152:967–978.
 39. Treviño J, Perez N, Ramirez-Peña E, Liu Z, Shelburne SA, Musser JM, Sumby P. 2009. CovS simultaneously activates and inhibits the CovR-mediated repression of distinct subsets of group A *Streptococcus* virulence factor-encoding genes. *Infect. Immun.* 77:3141–3149.
 40. Bradley PP, Priebat DA, Christensen RD, Rothstein G. 1982. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *J. Invest. Dermatol.* 78:206–209.
 41. Green NM, Zhang S, Porcella SF, Nagiec MJ, Barbican KD, Beres SB, LeFebvre RB, Musser JM. 2005. Genome sequence of a serotype M28 strain of group A streptococcus: potential new insights into puerperal sepsis and bacterial disease specificity. *J. Infect. Dis.* 192:760–770.
 42. Nagiec MJ, Lei B, Parker SK, Vasil ML, Matsumoto M, Ireland RM, Beres SB, Hoe NP, Musser JM. 2004. Analysis of a novel prophage-encoded group A *Streptococcus* extracellular phospholipase A2. *J. Biol. Chem.* 279:45909–45918.
 43. Turner CE, Kurupati P, Jones MD, Edwards RJ, Sriskandan S. 2009. Emerging role of the interleukin-8 cleaving enzyme SpyCEP in clinical *Streptococcus pyogenes* infection. *J. Infect. Dis.* 200:555–563.
 44. Sumby P, Whitney AR, Gravis EA, DeLeo FR, Musser JM. 2006. Genome-wide analysis of group A streptococci reveals a mutation that modulates global phenotype and disease specificity. *PLoS Pathog.* 2:e5. doi:10.1371/journal.ppat.0020005.
 45. Bakleh M, Wold LE, Mandrekar JN, Harmsen WS, Dimashkieh HH, Baddour LM. 2005. Correlation of histopathologic findings with clinical outcome in necrotizing fasciitis. *Clin. Infect. Dis.* 40:410–414.
 46. Cockerill FR, Thompson RL, Musser JM, Schlievert PM, Talbot J, Holley KE, Harmsen WS, Ilstrup DM, Kohner PC, Kim MH, Frankfort B, Manahan JM, Steckelberg JM, Roberson F, Wilson WR. 1999. Molecular, serological, and clinical features of 16 consecutive cases of invasive streptococcal disease. *Clin. Infect. Dis.* 26:1448–1458.
 47. Taylor FB, Bryant AE, Blick KE, Hack E, Jansen PM, Kosanke SD, Stevens DL. 1999. Staging of the baboon response to group A streptococci administered intramuscularly: a descriptive study of the clinical symptoms and clinical chemical response patterns. *Clin. Infect. Dis.* 29:167–177.
 48. Hermoso JA, Lagartera L, González A, Stelter M, García P, Martínez-Ripoll M, García JL, Menéndez M. 2005. Insights into pneumococcal pathogenesis from the crystal structure of the modular teichoic acid phosphorylcholine esterase Pce. *Nat. Struct. Mol. Biol.* 12:533–538.