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# 2-O-Sulfated Domains in Syndecan-1 Heparan Sulfate Inhibit Neutrophil Cathelicidin and Promote *Staphylococcus aureus* Corneal Infection\*

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**Background:** Syndecan-1 promotes bacterial infections, but how this is accomplished remains unclear.

**Results:** Syndecan-1 and 2-O-sulfated heparan compounds specifically enhanced *S. aureus* corneal virulence and inhibited bacterial killing by CRAMP secreted from degranulated neutrophils.

**Conclusion:** Specific structural motifs in syndecan-1 HS promote *S. aureus* corneal infection by inhibiting neutrophil CRAMP.

**Significance:** This study uncovers a new pathogenic role for syndecan-1 in bacterial infection.

Ablation of syndecan-1 in mice is a gain of function mutation that enables mice to significantly resist infection by several bacterial pathogens. Syndecan-1 shedding is induced by bacterial virulence factors, and inhibition of shedding attenuates bacterial virulence, whereas administration of purified syndecan-1 ectodomain enhances virulence, suggesting that bacteria subvert syndecan-1 ectodomains released by shedding for their pathogenesis. However, the pro-pathogenic functions of syndecan-1 ectodomain have yet to be clearly defined. Here, we examined how syndecan-1 ectodomain enhances *Staphylococcus aureus* virulence in injured mouse corneas. We found that syndecan-1 ectodomain promotes *S. aureus* corneal infection in an HS-dependent manner. Surprisingly, we found that this pro-pathogenic activity is dependent on 2-O-sulfated domains in HS, indicating that the effects of syndecan-1 ectodomain are structure-based. Our results also showed that purified syndecan-1 ectodomain and heparan compounds containing 2-O-sulfate motifs inhibit *S. aureus* killing by antimicrobial factors secreted by degranulated neutrophils, but does not affect intracellular phagocytic killing by neutrophils. Immunodepletion of antimicrobial factors with staphylocidal activities demonstrated that CRAMP, a cationic antimicrobial peptide, is primarily responsible for *S. aureus* killing among other factors secreted by degranulated neutrophils. Furthermore, we found that purified syndecan-1 ectodomain and heparan compounds containing 2-O-sulfate units potently and specifically inhibit *S. aureus* killing by synthetic CRAMP. These results provide compelling evidence that a specific subclass of sulfate groups, and not the overall charge of HS, permits syndecan-1 ectodomains to promote *S. aureus* corneal infection by inhibiting a key arm of neutrophil host defense.

Corneal diseases, including microbial keratitis, blind nearly 9 million people worldwide and are the second leading cause of blindness after cataracts (1). By one estimate, the annual incidence of microbial keratitis is ~500,000 patients worldwide and 30,000 patients in the United States alone (2). In addition to being a major cause of blindness, microbial keratitis is also associated with significant ocular morbidity, such as scarring and reduced visual acuity (3, 4). The major bacterial pathogens that cause keratitis are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Streptococcus pneumoniae* (5–7). One of the major challenges in ocular surface biology remains the ability to define how pathogens interact with host components and modulate or co-opt their activities to promote their survival in the corneal environment.

Many microbial pathogens, including viruses, bacteria, and parasites, are thought to exploit the heparan sulfate (HS)<sup>2</sup> moiety of HS proteoglycans (HSPGs) to infect host cells and to evade immune mechanisms (8, 9). HSPGs are expressed ubiquitously on the cell surface and in the extracellular matrix. HSPGs are comprised of one or several HS chains attached covalently to specific core proteins (10). HS binds to and regulates many molecules that have been implicated in the host defense against infectious agents, including cytokines, chemokines, and cationic antimicrobial factors (8, 9).

HS chains are unbranched polysaccharides comprised of repeating disaccharide units of hexuronic acid, either glucuronic (GlcA) or iduronic acid (IdoA), alternating with an unsubstituted or *N*-substituted glucosamine on which the substituents are either acetate or sulfate (11–13). In HSPG biosynthesis, a non-sulfated HS precursor is polymerized on specific

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<sup>2</sup> The abbreviations used are: HS, heparan sulfate; HP, heparin; 2ODS-HP, 2-O-desulfated HP; 6ODS-HP, 6-O-desulfated HP; fNLP, formyl-norleucine-leucine-phenylalanine; GlcA, glucuronic acid; GlcA2S, 2-O-sulfated glucuronic acid; H, heparosan; HSPG, heparan sulfate proteoglycan; IdoA, iduronic acid; IdoA2S, 2-O-sulfated iduronic acid; NS-H, *N*-sulfated heparosan; NS2OS-H, *N*- and 2-O-sulfated heparosan; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; *Sdc1*<sup>−/−</sup>, syndecan-1 null; *Sdc4*<sup>−/−</sup>, syndecan-4 null; TSB, tryptic soy broth.

serine residues of HSPG core proteins and then extensively modified by *N*-deacetylase/*N*-sulfotransferases, C5 epimerase, 2-*O*-sulfotransferase (2OST), 6OSTs, and 3OSTs in the Golgi. Unique sulfation patterns of HS are thought to dictate how HSPGs bind to molecules and regulate biological processes, including HS interactions with infectious agents. For example, envelope glycoproteins E1 and E2 of hepatitis C virus require and *N*- and 6-*O*-sulfate groups for efficient interaction with HS (14), whereas OmcB of *Chlamydia trachomatis* binds to 6-*O*-sulfated HS domains (15). These observations suggest that microbes target specific HS modifications to promote their pathogenesis, but whether GAG modifications are indeed important *in vivo* has yet to be determined. In fact, our knowledge of the role of HSPGs in infections is mostly derived from cell-based experiments performed *in vitro*, and their physiological significance, relevance, and function in infectious diseases remain largely speculative.

A growing body of evidence suggests that bacterial pathogens subvert syndecan-1 shedding to promote their pathogenesis in various tissues. Syndecan-1 is the predominant HSPG of epithelial cells, a cell type that most bacteria first encounter during their pathogenesis (8). *S. aureus* (16), *P. aeruginosa* (17), and *S. pneumoniae* (18) induce the shedding of syndecan-1 ectodomains from the cell surface through specific virulence factors in cultured epithelial cells. Moreover, in mice, syndecan-1 ablation is a gain of function of mutation where the syndecan-1 null (*Sdc1*<sup>-/-</sup>) mice are significantly protected from *P. aeruginosa* (19) and *S. aureus* (20) lung infection, and *S. aureus* corneal infection (21) compared with wild type (Wt) mice, suggesting that syndecan-1 shedding promotes bacterial pathogenesis. Indeed, inhibition of shedding reduces bacterial virulence, whereas administration of purified syndecan-1 ectodomains or HS, but not other glycosaminoglycans or syndecan-1 core protein devoid of HS, enhances bacterial virulence in mouse models of infection (19, 21). These results indicate that syndecan-1 ectodomains promote bacterial pathogenesis in an HS-dependent manner, but precisely how this is accomplished is incompletely understood.

Here, we examined how HS chains of syndecan-1 ectodomains enhance *S. aureus* virulence in injured corneal tissues. Our results surprisingly showed that 2-*O*-sulfated domains in syndecan-1 HS promote *S. aureus* corneal infection. Our study also showed that the antimicrobial peptide CRAMP secreted by degranulated neutrophils selectively kills *S. aureus*, and 2-*O*-sulfate motifs in syndecan-1 HS potently and specifically inhibit the staphylocidal activity of CRAMP. These results reveal a new pathogenic role for discrete domains in syndecan-1 HS in infection.

## Experimental Procedures

**Materials**—281-2 rat anti-mouse syndecan-1 ectodomain and Ky8.2 rat anti-mouse syndecan-4 monoclonal antibodies were purchased from BD Biosciences (San Jose, CA). Rabbit anti-mouse myeloperoxidase (L607) was from Cell Signaling (Danvers, MA) and goat anti-lactoferrin (C-15) and mouse anti-CRAMP (G-1) antibodies were from Santa Cruz Biotechnology (Dallas, TX). Mouse IgG was from Equitech Bio (Kerrville, TX). *S. aureus* BioParticles Opsonizing Reagent, Live/Dead

BacLight Bacterial Viability Kit, Alexa Fluor 488 and 594 Antibody Labeling Kits were obtained from Invitrogen (Carlsbad, CA). Chondroitin sulfate A (CS), formyl-norleucine-leucine-phenylalanine peptide (fNLP), and cytochalasin D were from Sigma. CRAMP peptide was synthesized at institutional core programs or obtained from Anaspec (Fremont, CA). Percoll was from GE Healthcare Life Sciences (Pittsburgh, PA) and protein A-agarose and protein G-agarose beads were from Pierce. Syndecan-1 ectodomains were purified from the conditioned medium of normal mammary gland epithelial cells as described previously (21), whereas syndecan-4 ectodomains were partially purified from the conditioned medium by DEAE chromatography and sequential absorption to anti-syndecan-2 and -3 and anti-syndecan-1 affinity resins to immunodeplete other syndecans. The partially purified syndecan-4 preparation was determined to contain no syndecan-1, -2, and -3 and glypican-1 and -3 by dot immunoblotting. Porcine mucosal HS and heparin, and *N*-desulfated, 2-*O*-desulfated and 6-*O*-desulfated heparin were from Neoparin (Alameda, CA). Heparosan was purified from *E. coli* K5 and chemoenzymatically *N*-sulfated or *N*- and 2-*O*-sulfated as described (22). Briefly, *N*-sulfated heparosan was synthesized by incubating chemically deacetylated heparosan with *N*-sulfotransferase and the sulfate donor 3'-phosphoadenosine 5'-phosphosulfate (PAPS), whereas *N*- and 2-*O*-sulfated heparosan was made by incubating *N*-sulfated heparosan with recombinant 2-*O*-sulfotransferase and PAPS. Disaccharide composition analysis revealed that ~95% of the disaccharides in *N*-sulfated heparosan are comprised of -GlcA-GlcNS- units, whereas *N*- and 2-*O*-sulfated heparosan is composed of about 20% of -GlcA2S-GlcNS- and 80% of -GlcA-GlcNS- disaccharide units. All other materials were purchased from Sigma, Thermo Fisher Scientific (Waltham, MA), or VWR (Westchester, PA).

**Mice**—Except for experiments comparing the response of Wt, *Sdc1*<sup>-/-</sup> and *Sdc4*<sup>-/-</sup> mice on the C57BL/6J background to *S. aureus* corneal infection (Fig. 1B), mice on the BALB/c background were used in all experiments since BALB/c mice are considered to be more susceptible to *S. aureus* keratitis compared with BL/6 mice (23). Unchallenged *Sdc1*<sup>-/-</sup> mice on both the BALB/c and C57BL/6J backgrounds and unchallenged *Sdc4*<sup>-/-</sup> mice on the C57BL/6J background are healthy with normal growth, reproduction, tissue morphology, complete blood cell counts, and serum chemistry parameters (24–26). Both female and male *Sdc1*<sup>-/-</sup>, *Sdc4*<sup>-/-</sup>, and Wt mice were used at an age of 8–10 weeks. Mice were maintained in microisolator cages under specific pathogen-free conditions in a 12 h light/dark cycle and fed a basal rodent chow *ad libitum*. All animal experiments were approved by the Institutional Animal Care and Use Committee of Children's Hospital, and complied with federal guidelines for research with experimental animals.

**Mouse Model of *S. aureus* Corneal Infection**—*S. aureus* strains 8325–4 (16), P1 (27), USA300 (28), and Woods (29) were from our culture collection. *S. aureus* strains were grown to late log growth phase in tryptic soy broth (TSB), and the bacterial concentration was approximated by turbidity measurement at 600 nm. After washing, the concentration was adjusted to ~5 × 10<sup>8</sup> cfu/5 μl. The exact bacterial concentra-

tion in the inoculum was determined by plating out serial dilutions onto TSB agar plates immediately after preparation. A single vertical scratch was made with a 29-gauge needle in one of the corneas of each anesthetized mouse without penetrating beyond the superficial stroma. The other eye served as an uninjured control. A 5  $\mu$ l suspension of *S. aureus* was applied topically to injured or uninjured corneas, and test reagents were administered topically at the indicated times. The dose of test compounds was selected on preliminary titration experiments. At 7–10 h post-infection, mice were euthanized and the bacterial burden in isolated corneas was determined. Isolated corneas were homogenized in TSB containing 0.1% (v/v) Triton X-100 and serial dilutions of homogenates were plated onto TSB agar plates.

*S. aureus* Killing by Neutrophils, Degranulated Neutrophil Extracts, and CRAMP—Neutrophils were isolated from bone marrows by Percoll gradient density centrifugation as described previously (21). Briefly, isolated femurs and tibias were cleaned and flushed with Hank's Balanced Salt Solution (HBSS) containing 10 mM HEPES, pH 7.4 (HBSS/HEPES). Bone marrow cells were centrifuged at  $300 \times g$  for 10 min, resuspended in 45% Percoll solution, layered on top of a 62 and 81% Percoll gradient solution, and centrifuged at  $1500 \times g$  for 30 min. The neutrophil layer between 62 and 81% Percoll was collected, washed, resuspended in HBSS/HEPES, and counted. Neutrophils were incubated with pre-opsonized *S. aureus* Woods strain in HBSS/HEPES containing 5% mouse serum for 2 h at 37 °C. Bacterial killing was enumerated by incubating test samples with 0.1% Triton X-100 in HBSS/HEPES for 30 min and plating serial dilutions onto TSB agar plates. To prepare degranulated neutrophil extracts, isolated neutrophils were stimulated with 1  $\mu$ M fNLP for 1 h at 37 °C, and the supernatants were collected by centrifugation at  $300 \times g$  for 5 min. *S. aureus* killing by fNLP-stimulated neutrophils supernatants or synthetic CRAMP was determined by incubating *S. aureus* with supernatants or CRAMP peptide at the indicated doses for 2 h at 37 °C and plating serial dilutions of the test samples onto TSB agar plates.

*Histology*—Eyes were enucleated from uninfected Wt or infected Wt and *Sdc1*<sup>−/−</sup> mice at 7 h post-infection, fixed in 4% paraformaldehyde/PBS for 4 h at room temperature, embedded in paraffin, and sectioned horizontally. Eye sections (5  $\mu$ m) were stained with Gram solution or immunostained with 281–2 anti-mouse syndecan-1 or Ky8.2 anti-mouse syndecan-4 monoclonal antibodies directly conjugated to Alexa 594 or 488. Stained tissue sections were visualized with a Zeiss Axiovert 40 CFL microscope and pictures were taken with the AxioCam MRm high resolution camera. Adobe Photoshop CS6 was used to process the acquired images.

*Statistical Analyses*—All data are expressed as mean plus or minus S.E. Differences between experimental and respective control groups were examined by Student's *t* test, and *p* values less than 0.05 were considered statistically significant.

## Results

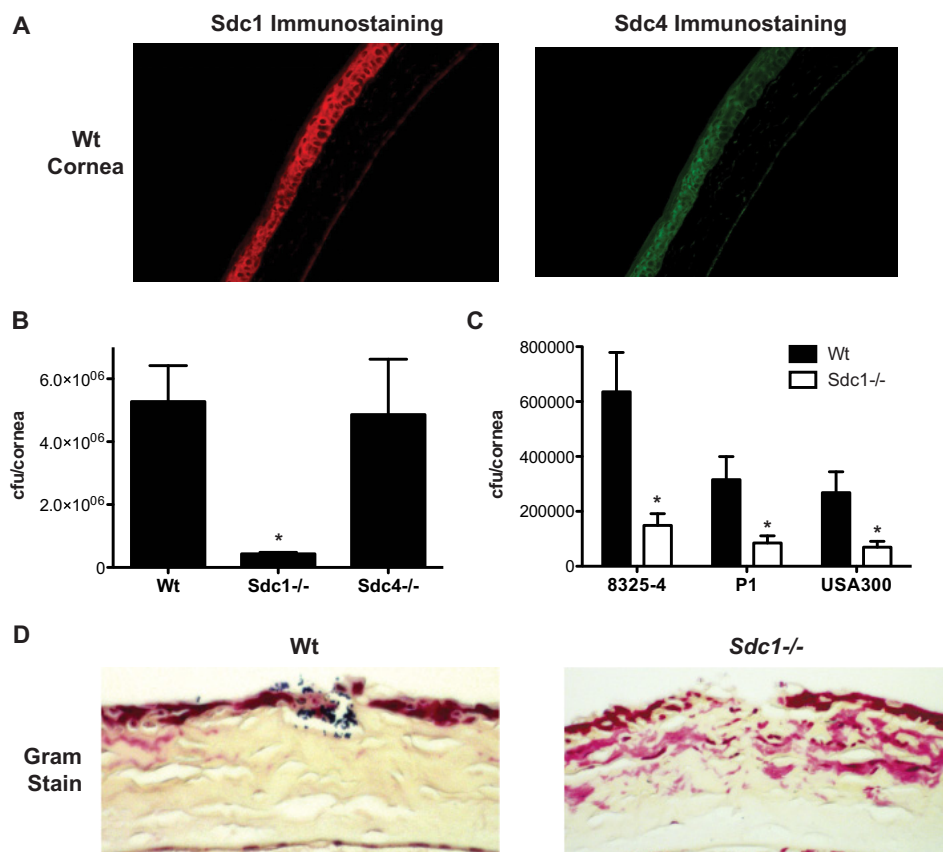
*Corneal Epithelial Syndecan-1 Specifically Promotes S. aureus Corneal Infection in an HS-dependent Manner*—We initially examined the expression of syndecans in the cornea.

Uninfected Wt corneas showed strong expression of syndecan-1 in the epithelium and weak expression in the endothelium (Fig. 1A). Syndecan-4 was expressed in a similar pattern in the corneal epithelium and endothelium, albeit at a lower level than that of syndecan-1 (Fig. 1A). Syndecan-2 was weakly expressed by keratocytes in the corneal stroma and syndecan-3 expression was not detected (data not shown). Based on these results, we examined whether syndecan-1 functions specifically in bacterial keratitis by comparing the response of Wt, *Sdc1*<sup>−/−</sup>, and *Sdc4*<sup>−/−</sup> mice to *S. aureus* corneal infection. Mouse corneas were injured with a single vertical scratch using a 29-gauge needle without penetrating beyond the superficial stroma and infected topically with  $5 \times 10^8$  cfu of *S. aureus* strain 8325–4. The corneal bacterial burden was quantified by plating out serial dilutions of infected corneal homogenates. The infectious burden was significantly reduced by over 10-fold in *Sdc1*<sup>−/−</sup> corneas compared with those of Wt and *Sdc4*<sup>−/−</sup> corneas, but similar between Wt and *Sdc4*<sup>−/−</sup> corneas (Fig. 1B), indicating that ablation of syndecan-1 enables mice to specifically resist *S. aureus* corneal infection.

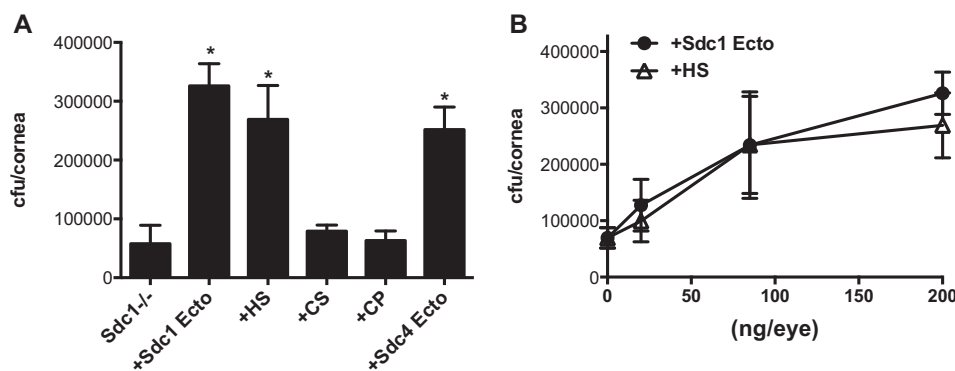
We next examined if the protection from *S. aureus* infection by syndecan-1 ablation is restricted to the 8325–4 strain by comparing the response of Wt and *Sdc1*<sup>−/−</sup> corneas to infection by 8325–4, P1 (clinical blood isolate), and USA300 (MRSA) strains. Although there were differences in the virulence of each bacterial strain, *Sdc1*<sup>−/−</sup> corneas were significantly protected from infection by all 3 strains tested compared with Wt corneas (Fig. 1C). Furthermore, reinforcing the prevailing view that injured areas are exclusively infected in the model of scarified corneal infection, Gram staining showed an intense accumulation of Gram-positive *S. aureus* at sites where the epithelium was scratched in Wt corneas, but not in *Sdc1*<sup>−/−</sup> corneas (Fig. 1D). Thus, despite observations suggesting that ablation of syndecan-1 delays wound healing in the cornea and skin (30), and that impairment of wound healing is a major risk factor for infection, these data suggest that syndecan-1 both specifically and prominently promotes *S. aureus* corneal infection.

Next we examined how syndecan-1 specifically enhances *S. aureus* virulence and if certain structural components of syndecan-1 HS are important by comparing the effects of purified syndecan-1 ectodomains, HS, CS, syndecan-1 core protein devoid of both HS and CS chains, and partially purified syndecan-4 ectodomains devoid of other syndecans including syndecan-1. Because syndecan-1 shedding is maximal at 3 h after *S. aureus* corneal infection (21), we administered purified ectodomains and related compounds at 3 h post-infection and quantified the corneal bacterial burden 7 h later. Both purified ectodomain and HS significantly increased the bacterial burden in *Sdc1*<sup>−/−</sup> corneas by 4- and 3-fold, respectively, whereas CS and core protein did not (Fig. 2A). Purified syndecan-1 ectodomain and HS also similarly enhanced *S. aureus* virulence in *Sdc1*<sup>−/−</sup> corneas in a dose-dependent manner (Fig. 2B). Incubation of bacteria with purified ectodomain or HS had no effect on bacterial growth *in vitro* (not shown). Interestingly, partially purified syndecan-4 ectodomains lacking syndecan-1 also significantly increased the corneal bacterial load (Fig. 2A), indicating that other HSPGs also possess critical HS motifs that





**FIGURE 1. Syndecan-1 is the predominant HSPG in the mouse corneal epithelium and *Sdc1*<sup>-/-</sup> mice specifically resist *S. aureus* corneal infection compared with Wt mice.** A, eye sections (5  $\mu$ m) of uninfected Wt mice were immunostained with 5  $\mu$ g/ml 281-2 anti-mouse syndecan-1 ectodomain or 5  $\mu$ g/ml Ky8.2 anti-mouse syndecan-4 ectodomain antibodies directly conjugated to Alexa 594 or 488 (original magnification,  $\times 200$ ). B, scarified corneas of Wt, *Sdc1*<sup>-/-</sup>, and *Sdc4*<sup>-/-</sup> mice on the BL/6J background were infected topically with  $5 \times 10^8$  cfu of *S. aureus* strain 8325-4. The corneal bacterial burden was quantified 7 h after infection. Data shown are mean  $\pm$  S.E. ( $n = 10$  in Wt,  $n = 12$  in *Sdc1*<sup>-/-</sup>, and  $n = 14$  in the *Sdc4*<sup>-/-</sup> groups, \*,  $p < 0.05$  relative to Wt, Student's  $t$  test). C, scarified corneas of Wt and *Sdc1*<sup>-/-</sup> mice on the BALB/c background were infected topically with  $5 \times 10^8$  cfu of *S. aureus* 8325-4, P1 or USA300, and the corneal bacterial burden was quantified at 7 h post-infection. Data shown are mean  $\pm$  S.E. (8325-4:  $n = 8$  for both Wt and *Sdc1*<sup>-/-</sup>, P1:  $n = 14$  for both Wt and *Sdc1*<sup>-/-</sup>, and USA300:  $n = 14$  for Wt and  $n = 12$  for *Sdc1*<sup>-/-</sup>; \*,  $p < 0.05$  relative corresponding Wt). D, eye sections (5  $\mu$ m) of Wt and *Sdc1*<sup>-/-</sup> mice isolated at 7 h after infection with *S. aureus* 8325-4 were Gram-stained (original magnification,  $\times 200$ ).

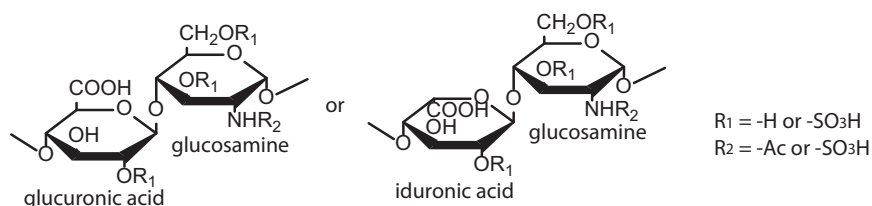


**FIGURE 2. Syndecan-1 ectodomain HS specifically promotes *S. aureus* corneal infection in a dose-dependent manner.** A, scarified corneas of *Sdc1*<sup>-/-</sup> mice were infected topically with  $5-6 \times 10^8$  cfu of *S. aureus* 8325-4, administered vehicle (*Sdc1*<sup>-/-</sup>, control) or 200 ng of purified syndecan-1 ectodomain, HS, CS, core protein (CP), or partially purified syndecan-4 ectodomain at 3 h after infection, and the corneal bacterial burden was quantified at 10 h after infection ( $n = 16$  for the control group,  $n = 10$  for the syndecan-1 ectodomain, HS and syndecan-4 ectodomain groups,  $n = 4$  for the CS and CP groups; \*,  $p < 0.05$  relative to control). B, scarified *Sdc1*<sup>-/-</sup> corneas were infected topically with  $5 \times 10^8$  cfu of *S. aureus*, administered increasing doses of purified syndecan-1 ectodomain or HS at 3 h post-infection, and the corneal bacterial burden was measured at 10 h post-infection ( $n = 4$  for 20 ng,  $n = 6$  for 85 ng, and  $n = 10$  for 200 ng groups for both ectodomain and HS).

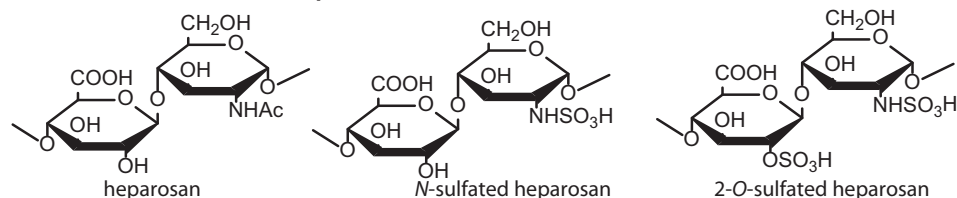
enhance *S. aureus* corneal virulence. More importantly, these data suggest that syndecan-1 functions specifically in *S. aureus* corneal infection because it is shed specifically by *S. aureus* infection.

**2-O-Sulfated Domains in Syndecan-1 HS Promote *S. aureus* Corneal Infection**—We next examined if a specific sulfate modification is important for HS's ability to enhance *S. aureus* virulence in the cornea. Structures of disaccharide repeating units

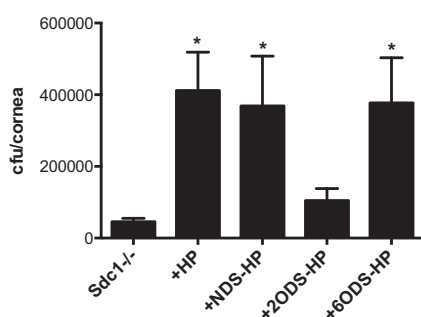
**A. Chemical structure of HS**



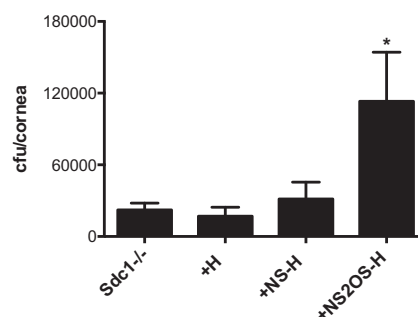
**B. Chemical structure of heparosan and derivatives**



**C**



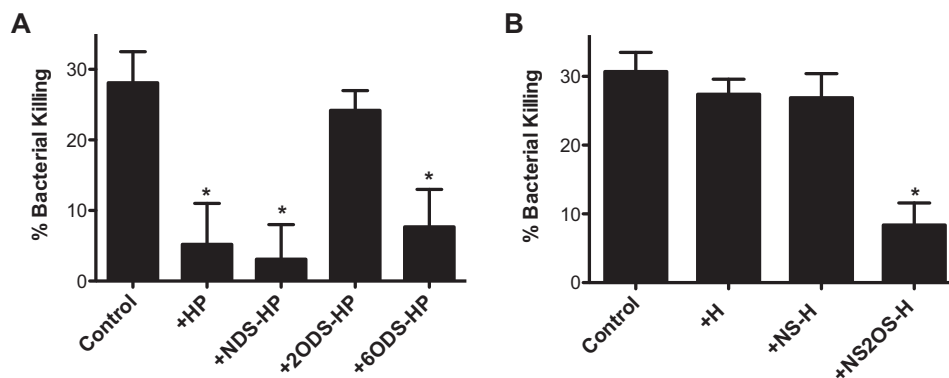
**D**



**FIGURE 3. Topical administration of 2-O-sulfated heparan compounds enhances *S. aureus* virulence in injured corneas.** A, diagram of chemical structures of repeating disaccharide units of HS. B, chemical structures of repeating disaccharide units of heparosan and derivatives. C, scarified corneas of *Sdc1*<sup>-/-</sup> mice were infected topically with  $3 \times 10^8$  cfu of *S. aureus* (*Sdc1*<sup>-/-</sup>, control), administered 200 ng of heparin (HP), N-desulfated HP (NDS-HP), 2-O-desulfated HP (2ODS-HP), or 6-O-desulfated HP (6ODS-HP) at 3 h post-infection, and the corneal bacterial burden was quantified at 10 h after infection ( $n = 6$  in all groups; \*,  $p < 0.05$  relative to control). D, scarified *Sdc1*<sup>-/-</sup> corneas were infected topically with  $3 \times 10^8$  cfu of *S. aureus* without (*Sdc1*<sup>-/-</sup>, control), administered 500 ng of heparosan (H), N-sulfated H (NS-H), or N- and 2-O-sulfated H (NS2OS-H) at 3 h post-infection, and the corneal bacterial burden was measured at 10 h post-infection ( $n = 6$  in all groups; \*,  $p < 0.05$  relative to control). The dose of HP and H compounds was based on preliminary titration experiments.

of HS are shown in Fig. 3A. HS primarily exists in nature as an extended helical structure and does not fold into tertiary structures (31). HS functions are largely specified by how it displays its highly negatively charged sulfate and to a lesser extent also its carboxyl motifs. For example, 2-O-sulfate groups of HS are essential in chick limb bud development and outgrowth (32), in triglyceride-rich lipoprotein clearance by hepatocytes (33), and in mammary ductal branching (34) in mice. We first tested the effects of chemically desulfated heparin compounds on *S. aureus* virulence in the cornea (Fig. 3C). Similar to purified syndecan-1 ectodomain and HS, topical administration of heparin significantly increased the bacterial burden in *Sdc1*<sup>-/-</sup> corneas compared with control *Sdc1*<sup>-/-</sup> corneas that received bacteria only. Administration of N- or 6-O-desulfated heparin also similarly increased the corneal bacterial burden. On the other hand, removal of 2-O-sulfate groups significantly inhibited the ability of heparin to increase *S. aureus* virulence in the cornea, suggesting that N- and 6-O-sulfation are dispensable, whereas 2-O-sulfation is critical for this activity. These data also suggest that the overall net charge of syndecan-1 HS is not the sole determinant of activity in *S. aureus* corneal infection since 2-O-desulfated heparin has a higher net charge than N-desulfated heparin.

Because chemical desulfation is selective, but not specific, we next tested the effects of heparosan and heparosan compounds that were sulfated *in vitro* (Fig. 3B). Heparosan is a capsular polysaccharide from bacteria that has a repeating disaccharide unit of -GlcA-GlcNAc-, and is essentially identical in structure to unmodified HS. To confirm that 2-O-sulfate groups are important, we synthesized N-sulfated and N- and 2-O-sulfated heparosan using a chemoenzymatic approach (22), and tested their effects on *S. aureus* virulence in the cornea. We used N-sulfated heparosan as a substrate for 2-O-sulfation because N-sulfation is necessary to efficiently sulfate heparosan at the 2-O-position. Topical administration of unmodified heparosan or N-sulfated heparosan had no significant effect on the corneal bacterial burden in *Sdc1*<sup>-/-</sup> mice, whereas administration of N- and 2-O-sulfated heparosan significantly increased the corneal bacterial burden by over 5-fold compared with mice that were infected with bacteria only (Fig. 3D). Heparin or heparosan compounds had no effect on bacterial growth *in vitro*. Altogether, these data indicate that 2-O-sulfated domains in syndecan-1 HS promote *S. aureus* corneal infection, and suggest that they do so by modulating the host response to infection.



**FIGURE 4. 2-O-sulfated domains in HS inhibit the staphylocidal activity of neutrophils.** A, Wt neutrophils ( $5 \times 10^5$ ) were incubated with  $2 \times 10^3$  cfu of pre-opsonized *S. aureus* in the absence (control) or presence of 3  $\mu\text{g}/\text{ml}$  HP, NDS-HP, 2ODS-HP, or 6ODS-HP for 2 h at 37 °C. Percent bacterial killing was enumerated by plating serial dilutions of detergent lysates onto TSB agar plates ( $n = 4$ , \*,  $p < 0.05$  relative to control). B, Wt neutrophils ( $5 \times 10^5$ ) were incubated with  $2 \times 10^3$  cfu of pre-opsonized *S. aureus* in the absence (control) or presence of 10  $\mu\text{g}/\text{ml}$  H, NS-H, or NS2OS-H for 2 h at 37 °C and bacterial killing was determined ( $n = 8$ , \*,  $p < 0.05$  relative to control). The dose of HP and H compounds was selected on preliminary titration experiments with HP or NS2OS-H.

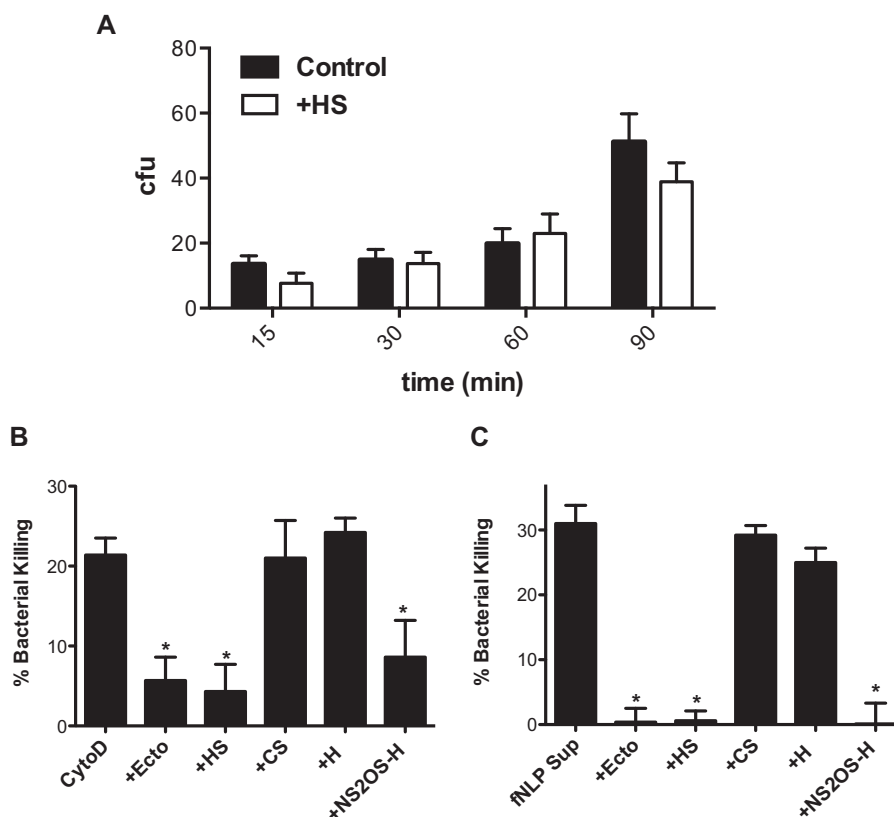
**2-O-Sulfated Domains in Syndecan-1 HS Inhibit the Extracellular Killing of *S. aureus* by Neutrophils**—Neutrophils are rapidly deployed to sites of infection and injury, and are indispensable in the innate host defense against the majority of acute bacterial infections, including bacterial keratitis (35, 36). Consistent with these observations, immunodepletion of neutrophils significantly increased *S. aureus* virulence in *Sdc1*<sup>−/−</sup> corneas (21), indicating that neutrophils are essential to rapidly clear *S. aureus* and enable *Sdc1*<sup>−/−</sup> mice to resist *S. aureus* corneal infection. However, neutrophils do not express syndecan-1 and do not bind to syndecan-1. Syndecan-1 also does not regulate neutrophil influx into corneas infected with *S. aureus*, and isolated neutrophils of both *Sdc1*<sup>−/−</sup> and Wt mice similarly kill *S. aureus* (21). Moreover, Wt and *Sdc1*<sup>−/−</sup> neutrophils are similar in size, granularity, and pattern of GR1 staining, *Sdc1*<sup>−/−</sup> neutrophils do not have an inherent defect in their ability to migrate, and *Sdc1*<sup>−/−</sup> mice contain normal numbers of circulating neutrophils (25, 37). Instead, syndecan-1 ectodomains have been shown to promote *S. aureus* corneal infection by inhibiting neutrophil-mediated *S. aureus* killing in an HS-dependent manner (21).

Thus, we next explored whether specific structural features of syndecan-1 HS inhibit the host defense activities of neutrophils against *S. aureus*. We first examined the effects of chemically desulfated heparin and chemoenzymatically sulfated heparosan on the killing of pre-opsonized *S. aureus* by Wt neutrophils isolated from the bone marrow. Heparin significantly inhibited *S. aureus* killing by neutrophils, and removal of sulfate groups at the *N*- or 6-*O*-position had no effect on this inhibitory activity (Fig. 4A). However, 2-*O*-desulfated heparin lost its capacity to inhibit *S. aureus* killing by neutrophils, suggesting that 2-*O*-sulfate groups are important. Consistent with these observations, heparosan and *N*-sulfated heparosan only slightly inhibited *S. aureus* killing by neutrophils, but the marginal difference (<4%) did not reach significance (Fig. 4B). In contrast, *N*- and 2-*O*-sulfated heparosan significantly inhibited the killing of *S. aureus* by neutrophils (Fig. 4B). At the doses tested, the heparin and heparosan compounds did not affect the viability of isolated neutrophils. These findings suggest that 2-*O*-sulfated domains in syndecan-1 HS promote *S. aureus* corneal infection by inhibiting bacterial killing by neutrophils.

Neutrophils can kill bacteria by extracellular or intracellular phagocytic mechanisms. Most of the antimicrobial activity of neutrophils are thought to occur within intracellular phagosomes, but extracellular killing mechanisms involving antimicrobial factors secreted by neutrophil degranulation or released and embedded in neutrophil extracellular traps also comprise an important arm of host defense against infections (36). To determine how 2-*O*-sulfated domains in syndecan-1 HS inhibit *S. aureus* killing by neutrophils, we first examined the effects of HS on the rate of phagocytic killing. Wt neutrophils were incubated with pre-opsonized *S. aureus* in the absence or presence of HS for 15, 30, 60, or 90 min, incubated with gentamycin to kill extracellular bacteria, and the intracellular bacterial load was determined. The number of live intracellular bacteria was similar between the neutrophils incubated with bacteria only and with bacteria and HS at all times examined (Fig. 5A), indicating that HS does not inhibit phagocytic killing mechanisms of neutrophils. In fact, intracellular killing was slightly inhibited by HS at 90 min postincubation, although the difference did not reach significance (Fig. 5A).

Next, we examined the effects of purified syndecan-1 ectodomain and related compounds on the extracellular killing of *S. aureus* by neutrophils. Neutrophils were pre-treated with cytochalasin D to inhibit phagocytosis and incubated with *S. aureus* in the absence or presence of purified syndecan-1 ectodomain, HS, CS, heparosan, or *N*- and 2-*O*-sulfated heparosan. Purified ectodomain, HS, and *N*- and 2-*O*-sulfated heparosan significantly inhibited the extracellular killing of *S. aureus* by neutrophils, whereas CS and heparosan did not (Fig. 5B). Together with the data from the phagocytic killing assay, these findings suggest that 2-*O*-sulfated domains in syndecan-1 HS promote *S. aureus* corneal infection by inhibiting antibacterial factors secreted from neutrophils.

We further explored this hypothesis by examining the effects of purified syndecan-1 ectodomain and heparan compounds on the antibacterial activity of degranulated contents collected from neutrophils stimulated with the formylated tripeptide, formyl-norleucine-leucine-phenylalanine (fNLP). Neutrophils express heterogeneous granules and vesicles that contain various peptides and proteins with antimicrobial activity (38). These granules and vesicles are differentially released upon



**FIGURE 5. 2-O-sulfated domains in syndecan-1 HS inhibit extracellular killing mechanisms of neutrophils.** A, Wt neutrophils ( $5 \times 10^5$ ) were incubated with pre-opsonized *S. aureus* for 15, 30, 60, or 90 min in the absence or presence of 3  $\mu$ g/ml HS, washed, incubated with 100  $\mu$ g/ml gentamycin for 30 min to kill extracellular bacteria, washed, treated with TSB containing 0.1% (v/v) Triton X-100, and detergent lysates were plated out to determine the rate of phagocytic killing (mean  $\pm$  S.E.,  $n = 5$ ). B, Wt neutrophils ( $5 \times 10^5$ ) were pre-treated with 10  $\mu$ g/ml cytochalasin D, incubated with *S. aureus* ( $2 \times 10^3$  cfu) in the presence of cytochalasin D without or with 1  $\mu$ g/ml purified ectodomain, 3  $\mu$ g/ml HS or CS, or 10  $\mu$ g/ml H or NS2OS-H for 2 h at 37  $^{\circ}$ C, and bacterial killing was determined ( $n = 22$  for the cytochalasin D group,  $n = 11$  for the +Ecto group,  $n = 16$  for the +HS group,  $n = 4$  for +CS and +H groups, and  $n = 5$  for the +NS2OS-H group; \*,  $p < 0.05$  relative to the cytochalasin D group). C, supernatants from neutrophils stimulated with 1  $\mu$ M fNLP for 1 h were incubated with *S. aureus* ( $1.5 \times 10^3$  cfu) in the absence (fNLP sup) or presence of 1  $\mu$ g/ml ectodomain, 3  $\mu$ g/ml HS or CS, or 10  $\mu$ g/ml H or NS2OS-H for 2 h at 37  $^{\circ}$ C, and bacterial killing was determined ( $n = 5$  in all groups; \*,  $p < 0.05$  relative to the fNLP sup group).

neutrophil stimulation by both biological and chemical reagents, including microbial products (e.g. formylated peptides, glycolipids), cytokines, and chemokines, and phorbol esters. In general, granules formed during the late stages of neutrophil differentiation are more readily mobilized than granules formed during the early stages of differentiation (39). Stimulation by fNLP released both specific (lactoferrin, CRAMP) and azurophilic (myeloperoxidase) granule contents (not shown). Moreover, we found that fNLP-stimulated neutrophil extracts possess potent staphylocidal activity, which was significantly inhibited by purified ectodomain and 2-O-sulfated heparan compounds (HS, and *N*- and 2-O-sulfated heparosan), but not by those lacking 2-O-sulfate motifs (CS or unmodified heparosan) (Fig. 5C). Together, these data indicate that 2-O-sulfated domains in syndecan-1 HS inhibit *S. aureus* killing by degranulated neutrophil antimicrobial factors, and suggest that syndecan-1 HS inhibits this particular arm of extracellular killing mechanism to enhance *S. aureus* virulence in corneal tissues.

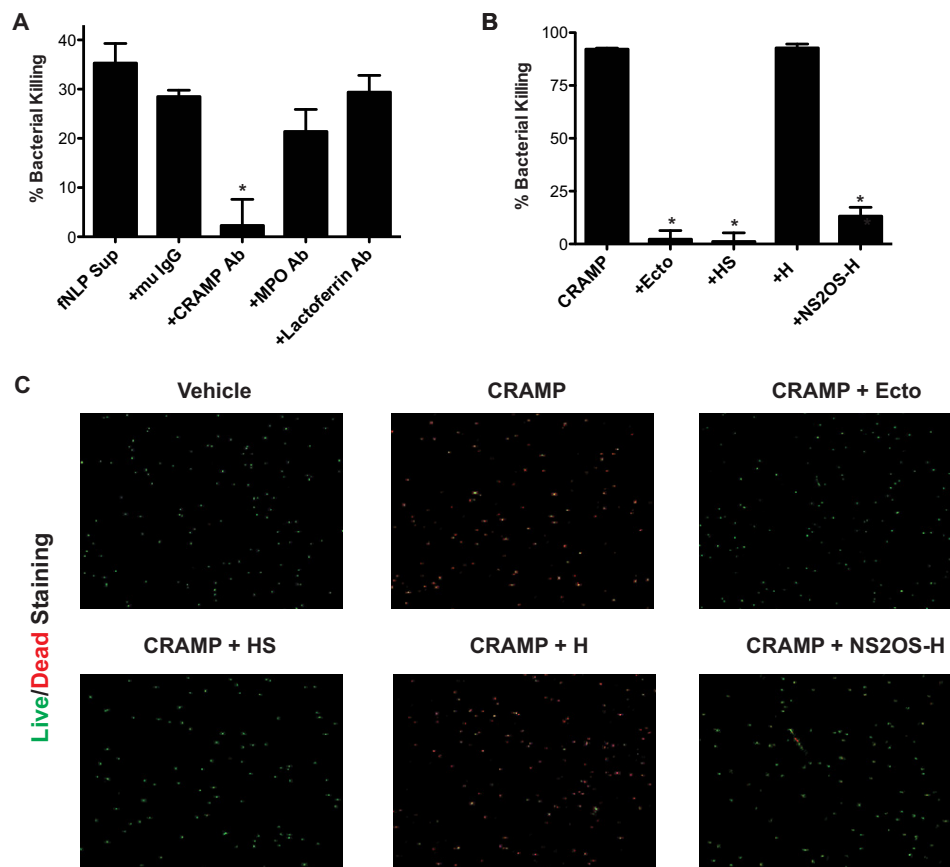
**Syndecan-1 HS Inhibit the Killing of *S. aureus* by CRAMP through Its 2-O-Sulfated Domains**—Next, to identify the degranulated neutrophil factor inhibited by syndecan-1 HS, we first examined the effects of immunodepleting several antimicrobial factors with anti-staphylococcal activity from fNLP-stimulated neutrophil supernatants on *S. aureus* killing.

Immunoprecipitation with nonspecific mouse IgG or immunodepletion of lactoferrin or myeloperoxidase did not inhibit *S. aureus* killing by degranulated neutrophil supernatants (Fig. 6A). On the other hand, immunodepletion of the cationic antimicrobial peptide CRAMP significantly inhibited the staphylocidal activity of degranulated neutrophil supernatants (Fig. 6A), suggesting that syndecan-1 HS inhibits CRAMP to inhibit *S. aureus* killing by neutrophils.

CRAMP kills *S. aureus* (40) and *S. aureus* killing by LL-37, the human homologue of CRAMP, is inhibited by HS (41, 42). Consistent with these reports, we found that incubation with synthetic CRAMP for 2 h kills over 90% of *S. aureus* (Fig. 6B). Importantly, purified syndecan-1 ectodomain, HS, and *N*- and 2-O-sulfated heparosan, but not unmodified heparosan without 2-O-sulfate groups, abolished or significantly inhibited *S. aureus* killing by CRAMP (Fig. 6B). These findings were also confirmed by a live/dead staining assay where most *S. aureus* cells incubated with CRAMP only or CRAMP and heparosan were dead (red), whereas the majority of those incubated with CRAMP and ectodomain, HS, or *N*- and 2-O-sulfated heparosan were alive (green) (Fig. 6C).

Together with data from the immunodepletion experiments, these data suggest that syndecan-1 HS specifically inhibits CRAMP in degranulated neutrophil extracts to promote





**FIGURE 6. Neutrophil extracellular killing of *S. aureus* is mediated by CRAMP, and syndecan-1 ectodomain and 2-O-sulfated heparan compounds inhibit killing of *S. aureus* by CRAMP.** A, supernatants from Wt neutrophils stimulated with fNLP were incubated with *S. aureus* ( $1.5 \times 10^3$  cfu) or were immunoprecipitated with mouse IgG, anti-CRAMP, anti-MPO, or anti-lactoferrin antibodies and then incubated with *S. aureus* for 2 h at 37 °C, and % bacterial killing was determined ( $n = 5$  in all groups; \*,  $p < 0.05$  compared with the fNLP sup group). B, CRAMP (10  $\mu$ g/ml) was pre-incubated with vehicle or 10  $\mu$ g/ml purified ectodomain, HS, H, or NS2OS-H for 15 min and then incubated with *S. aureus* ( $10^3$  cfu) for 2 h at 37 °C, and % bacterial killing was determined ( $n = 6$  in all groups; \*,  $p < 0.05$  compared with the CRAMP only group). C, *S. aureus* incubated with vehicle, CRAMP, or CRAMP and ectodomain, HS, H, or NS2OS-H were stained for live and dead bacteria.

*S. aureus* corneal infection. However, why syndecan-1 HS targets CRAMP and not other cationic antimicrobial peptides with anti-staphylococcal activity is not clear. Because most antimicrobial peptides and proteins kill *S. aureus* at relatively high doses, perhaps the concentration of other staphylocidal factors released from degranulated neutrophils by fNLP stimulation did not reach the effective dose. Also, since mouse neutrophils do not express defensins (43), which are inhibited by syndecan-1 HS (19), it is quite possible that syndecan-1 ectodomains may inhibit multiple cationic antimicrobial peptides in clinical *S. aureus* keratitis. Regardless, our data clearly show that 2-O-sulfated domains in syndecan-1 HS specifically and potently inhibit *S. aureus* killing by CRAMP, degranulated neutrophil extracts and intact neutrophils, and promote *S. aureus* corneal infection *in vivo*.

## Discussion

We report here that syndecan-1 is abundantly and selectively expressed in the corneal epithelium, and that HS chains of syndecan-1 promote *S. aureus* corneal infection in mice. While cell surface HSPGs are widely thought to promote infection by serving as attachment sites for pathogens, syndecan-1 does not bind directly to *S. aureus* and does not facilitate *S. aureus* adhesion to cultured corneal epithelial cells (21). Instead, syndecan-1

is shed from the cell surface during *S. aureus* corneal infection and the shed ectodomain enhances *S. aureus* virulence in corneal tissues. Our results here indicate that HS chains of syndecan-1 ectodomain promote pathogenesis by impeding bacterial clearance by neutrophils. We found that syndecan-1 ectodomain does not inhibit phagocytic killing of *S. aureus* by neutrophils, but that it significantly inhibits *S. aureus* killing by antimicrobial factors secreted by degranulated neutrophils. Our studies also showed that the antimicrobial peptide CRAMP is the primary target of syndecan-1 ectodomains in degranulated neutrophil extracts. Furthermore, our study showed that 2-O-sulfate groups in HS are essential for all of these pro-pathogenic activities of syndecan-1 ectodomain. Together, these observations suggest a new function of syndecan-1 in microbial pathogenesis where 2-O-sulfate motifs in its HS chains promote *S. aureus* corneal infection by inhibiting *S. aureus* killing by neutrophil CRAMP.

Our results showing that *Sdc1*<sup>-/-</sup> mice, but not *Sdc4*<sup>-/-</sup> mice, are significantly protected against *S. aureus* corneal infection suggest that syndecan-1 functions specifically in this infectious disease, despite similar cellular distribution patterns of syndecan-1 and -4 in the corneal epithelium. How this is accomplished is incompletely understood. Our data indicate

that both purified syndecan-1 ectodomains and partially purified syndecan-4 ectodomains devoid of other syndecans are capable of enhancing *S. aureus* corneal virulence. However, syndecan-1 is expressed at a higher level than that of syndecan-4 in the corneal epithelium, so the abundance of syndecan-1 may overwhelm the potential effects of syndecan-4 in *S. aureus* corneal infection. Furthermore,  $\alpha$ -toxin is a major virulence factor for *S. aureus* corneal infection (44, 45), and we previously found that  $\alpha$ -toxin stimulates the shedding of syndecan-1, but not syndecan-4 ectodomains in cell culture-based assays (16), suggesting that the specific functions of syndecan-1 in corneal tissues are also controlled by its susceptibility to  $\alpha$ -toxin-induced ectodomain shedding.

Alternatively, because our results suggest that discrete HS domains enhance *S. aureus* virulence in the cornea, syndecan-1 HS may contain unique structural features that enable it to function specifically in *S. aureus* corneal infection. However, opposing this idea are the findings that only minor structural and functional differences are found in HS chains of syndecan-1 and -4 in mouse mammary gland epithelial cells (46) and of syndecan-4 and glypicans in rat embryonic fibroblasts (47), suggesting that structural features of HS chains are cell type specific and not core protein specific. Taken together, these observations suggest that the prominent functions of syndecan-1 in *S. aureus* corneal infection are considered to be a reflection of its abundant expression and selective shedding by *S. aureus*  $\alpha$ -toxin.

Our results also showed that syndecan-1 HS does not affect intracellular phagocytic killing of *S. aureus* by neutrophils, but instead inhibits an extracellular killing mechanism of *S. aureus* by CRAMP secreted upon neutrophil degranulation. Because *S. aureus* possesses multiple mechanisms to evade intracellular phagosomal killing (48–50), perhaps *S. aureus* subverts syndecan-1 only to protect itself from extracellular killing by degranulated antimicrobial factors. CRAMP belongs to the cathelicidin family of antimicrobial peptides, which includes human LL-37, porcine PR-39, and bovine batenecins, among others (51). The importance of the cathelicidins in host defense is quite clear from both animal and cell-based infection studies. For example, LL-37 kills a wide variety of microbial pathogens, including viruses, bacteria and fungi, and is chemotactic for leukocytes (51). Knock-out mice lacking the CRAMP gene (*Cnlp*<sup>-/-</sup>) show increased morbidity or mortality in group A *Streptococcus* skin infection (52), *Klebsiella pneumoniae* lung infection (53), *P. aeruginosa* corneal infection (54), *E. coli* urinary tract infection (55), and *Citrobacter rodentium* intestinal infection (56). These observations suggest that despite the fact that antimicrobial peptide functions are considered largely redundant, the cathelicidins appear to be essential host defense factors in certain infectious diseases. Based on these observations, we propose that subversion of syndecan-1 HS to inhibit neutrophil CRAMP may be a critical virulence activity of *S. aureus* in the cornea.

It is important to note that CRAMP is not only expressed by neutrophils, but also by macrophages and epithelial cells (51), suggesting that syndecan-1 HS may also inhibit CRAMP produced by other cell types in *S. aureus* corneal infection. However, the observation that CRAMP is not expressed in injured

but uninfected mouse corneas (57) suggest that epithelial cell-derived CRAMP is not targeted for inhibition by syndecan-1, although epithelial cells are thought to make antimicrobial peptides on request. More importantly, we previously found that neutrophil depletion significantly enhances *S. aureus* virulence in the *Sdc1*<sup>-/-</sup> cornea (21), which provides additional evidence that neutrophil-derived, and not epithelial cell-derived CRAMP is important.

One of the major findings of our study was that 2-O-sulfated groups are essential for the ability of syndecan-1 HS to inhibit CRAMP- and neutrophil-mediated *S. aureus* killing, and to promote *S. aureus* corneal infection in mice. These observations were surprising because many HS activities are thought to depend more on the overall organization of HS domains and on the overall net charge of the glycosaminoglycan than on specific modifications (13). However, the structural basis of how HS regulates biological molecules and their processes is still being worked out. What is known for 2-O-sulfation is that it is essential for normal development since mice deficient in 2-O-sulfotransferase die few days after birth due to renal agenesis and CNS and skeletal abnormalities (58). Regulation of several growth factor activities by HS, such as FGF signaling and Wnt signaling (59, 60), and uptake of plasma lipoproteins by hepatocytes (33) have also been shown to be dependent on the presence of 2-O-sulfated uronic acids. However, how 2-O-sulfate groups in HS mediate the inhibition of neutrophil CRAMP-mediated host defense and enhance *S. aureus* virulence in the cornea have yet to be elucidated. Because HS binds to antimicrobial peptides and inhibit their antibacterial activity by interfering with peptide binding to target bacterial cells (19), syndecan-1 HS is also expected to inhibit CRAMP in a similar manner. A particular binding site in CRAMP may directly bind to 2-O-sulfated uronic acids in syndecan-1 HS or bind to a conformation of syndecan-1 HS that is dictated by 2-O-sulfated motifs. In fact, spacing of cationic amino acids is important for the anti-bacterial activity of LL-37 (61), suggesting that similar spacing of cationic residues in CRAMP may allow this peptide to avidly bind to 2-O-sulfate motifs in syndecan-1 HS.

Furthermore, the antibacterial activity of LL-37 is dependent on the extent of amphipathic  $\alpha$ -helicity (62). In water, however, LL-37 exhibits a disordered structure consistent with the fact that the energy provided by the hydrogen bonds are not sufficient to overcome the entropic energy associated with folding in short polypeptides, suggesting that the  $\alpha$ -helical content of cathelicidins increases when mobilized to kill pathogens. Considering the fact that hydrogen bonds of short peptide  $\alpha$ -helices are readily broken by water, perhaps binding of highly anionic molecules like syndecan-1 HS, which would accompany water, may disrupt the  $\alpha$ -helicity of CRAMP and hence its anti-staphylococcal activity. Alternatively or concurrently, syndecan-1 HS binding may prevent CRAMP oligomerization, which is considered important for cathelicidins to form toroidal pores in bacterial membranes (62). Additional studies are required to precisely determine how 2-O-sulfated domains in syndecan-1 HS inhibit CRAMP activity.

Another interesting finding of this study was that 2-O-sulfated heparosan inhibits CRAMP- and neutrophil-mediated *S. aureus* killing, and enhances *S. aureus* virulence in mouse

corneas. HS 2-O-sulfation can occur on both IdoA and GlcA, but is mostly found on IdoA in native HS and heparin due primarily to a higher affinity of 2-O-sulfotransferase for IdoA over GlcA (63). However, heparosan only contains GlcA, suggesting an intriguing possibility where 2-O-sulfated GlcA (GlcA2S)-containing domains in syndecan-1 HS mediate its pro-pathogenic activities in *S. aureus* corneal infection. IdoA can assume both a  ${}^4C_1$  chair and  ${}^2S_0$  skew boat forms as these have nearly identical energy conformations (64), allowing IdoA to bind to HS-binding ligands in either conformation. This conformational flexibility of IdoA is thought to be important for the ability of heparin and HS to interact with many proteins. In contrast, GlcA prefers a  ${}^4C_1$  conformation, giving a relatively rigid structural unit in HS and heparin and potentially mediate specific interactions with certain HS-binding ligands (64). Thus, syndecan-1 HS binding to CRAMP may be dictated by regions containing GlcA2S and that this interaction mediates the highly specific activities of syndecan-1 HS and 2-O-sulfated heparan compounds in *S. aureus* corneal infection. However, our results do not completely exclude the possibility that IdoA2S in the  ${}^4C_1$  chair conformation can function similarly.

In addition, although GlcA2S is a rare modification and the content of GlcA2S in corneal syndecan-1 HS is not known, it is worth noting that certain tissues, such as the liver (65) and brain (66), have a higher proportion of GlcA2S, suggesting a possibility where corneal epithelial tissues may also contain higher levels of this sulfate modification. Interestingly, an unusual *N*-unsubstituted and 3-O-sulfated glucosamine unit has been shown to mediate the specific binding of herpes simplex virus gD protein (67), suggesting that certain pathogens may subvert rare HS modifications in syndecan-1 for their pathogenesis.

In sum, our findings reveal a new pathogenic role for syndecan-1 in *S. aureus* corneal infection where its shed ectodomain inhibits neutrophil-mediated defense mechanisms crucial to the clearance of *S. aureus* in the cornea. Although the normal functions of syndecan-1 in the cornea remain to be elucidated, our findings suggest a possible beneficial role of inhibiting syndecan-1 HS and, in particular, the activity of 2-O-sulfated HS domains in treating *S. aureus* keratitis, for which new interventions are needed. Furthermore, CRAMP is not only important for host defense against *S. aureus*, but has also been shown to be critical in infections caused by other major pathogens of the ocular surface, such as *P. aeruginosa* (54) and *S. pneumoniae* (68). These observations suggest that neutralizing syndecan-1 HS to enhance CRAMP activity may be a viable option for the treatment of infectious keratitis caused by multiple bacterial pathogens of the ocular surface.

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