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Syndecan 1 Shedding Contributes to *Pseudomonas aeruginosa* Sepsis

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The innate immune system is comprised of many components that function coordinately to prevent bacterial sepsis. However, thermal injury suppresses many of these factors, and the opportunistic pathogen *Pseudomonas aeruginosa* takes advantage of this condition, making it one of the leading causes of morbidity and mortality in the setting of thermal injury. *P. aeruginosa* is extremely efficient at colonizing burn wounds, spreading systemically, and causing sepsis, which often results in a systemic inflammatory response, multiple-organ failure, and death. The pathogenicity of *P. aeruginosa* is due to the arsenal of virulence factors produced by the pathogen and the immunocompromised state of the host. Syndecan 1 is a major heparan sulfate proteoglycan present on many host cells involved in thermal injury. Syndecan 1 anchored to the cell surface can be cleaved in a process termed ectodomain shedding. Syndecan 1 shedding results in the release of intact, soluble proteoglycan ectodomains that have diverse roles in innate immunity. Here we show for the first time that thermal injury results in shedding of syndecan 1 from host tissue. Our data show that syndecan 1 null mice are significantly less susceptible to *P. aeruginosa* infection than their wild-type counterparts, as demonstrated by (i) significantly lower mortality; (ii) absence of systemic spread of *P. aeruginosa*; and (iii) significant reductions in some proinflammatory cytokines. These results suggest that shed syndecan 1 plays an important role in the pathogenesis of *P. aeruginosa* infection of thermal injury and that syndecan 1-neutralizing agents may be effective supplements to current *P. aeruginosa* treatments.

The gram-negative opportunistic pathogen *Pseudomonas aeruginosa* is particularly prevalent in burn wound infections. *P. aeruginosa* bacteremia is one of the leading causes of morbidity and mortality in the setting of thermal injury. It often causes multiorgan failure and results in mortality rates of up to 50% (2, 25, 26, 39). The extensive damage induced during *P. aeruginosa* infection is due to the ability of the microorganism to produce a large array of virulence factors (25) and the immunocompromised state of the individual. However, the increased ability of *P. aeruginosa* over many other pathogens to cause sepsis in thermally injured individuals is not fully understood.

Beyond the initial barrier of the skin, several components of the host's innate immune system are designed to act as sentinels that guard against systemic bacterial attacks. Toll-like receptors (TLRs) are transmembrane receptors found on host cells that recognize pathogen-associated molecular patterns (19). Upon recognition of a pathogen-associated molecular pattern, TLRs activate downstream signaling events, which induce an immediate inflammatory response (19). Acute inflammation, as seen in thermal injury, is characterized by vasodilatation and neutrophil infiltration. Consequences of inflammation include the production of cytokines and chemokines. Chemokines, such as interleukin-8 (IL-8), recruit neutrophils into the damaged area, which normally engulf the infecting bacteria before high numbers are reached. Proinflammatory cytokines, such as IL-1, IL-6, and tumor necrosis factor alpha (TNF-α), are produced predominately by activated macrophages. Among their many roles, cytokines regulate neutrophil functions and perpetuate the inflammatory response. Although these first lines of defense are normally effective, the highly devascularized environment of a burn wound and overall dysregulation of immunity in burned individuals provide an ideal setting for the proliferation and spread of bacteria within the injured tissue and subsequent dissemination throughout the immunocompromised host. In this environment, inflammation can shift from a protective process to a destructive one. The massive neutrophil infiltration, characteristic of thermal injury, may actually have a negative biological effect. Activated neutrophils release oxidants and hydrolytic enzymes that, when combined with bacterial degradative enzymes, can result in increased proteolysis within the wound and may actually aid spread and eventual dissemination of bacteria (14, 33). This neutrophil paradox has been implicated in the pathology of many chronic inflammatory conditions (36).

Dozens of inflammatory mediators play important roles in balancing the protective and destructive effects of inflammation. One important group of inflammatory mediators are heparan sulfate proteoglycans (HSPGs) (7). HSPGs are ubiquitous molecules expressed by all adherent cells and are composed of a core protein and one or more covalently attached heparan sulfate (HS) chains (7). The HS chains are made up of repeating units of hexuronic acid and N-substituted glucosamine disaccharide units. One family of HSPGs implicated in inflammation is the syndecans. Four syndecan family members (syndecan 1 to 4) have been cloned in mammals and are implicated in mediating a wide range of effects, including leukocyte rolling on endothelial cells, wound repair, angiogenesis, vascular permeability, and modulation of chemokine activity

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(7). Syndecans attached to the cell surface can be cleaved in a process termed shedding. Syndecan shedding results in the release of intact, soluble proteoglycan ectodomains into the host cell environment that can mediate cellular physiology at distant sites (7). Although syndecan ectodomains are shed at low levels as part of normal cell turnover, tissue injury can markedly activate syndecan shedding (12). Thus, shed syndecan 1 (Sdc-1) is not found at detectable levels in the biological fluids of healthy individuals, but high levels of shed Sdc-1 and Sdc-4 have been documented in human dermal wound fluids (37) and in tracheal aspirates of ventilated preterm infants (13).

Syndecan shedding during tissue injury is presumably due to mechanical shearing of the ectodomains and/or cleavage by host cell-derived proteases (12). However, it has also been shown that the LasA protease produced by *P. aeruginosa* activates syndecan 1 shedding (23). Shed Sdc-1 contributed to *P. aeruginosa* pathogenesis in a mouse model of lung infection (22). Sdc-1 knockout (Sdc-$1^{-/-}$) mice were significantly more resistant to *P. aeruginosa* lung infection than their wild-type (WT) counterparts, as demonstrated by decreased mortality and bacteremia (22). Furthermore, treating *P. aeruginosa*-infected WT mice with specific inhibitors of Sdc-1 shedding and HS was therapeutic (22). Considering the demonstrated importance of shed Sdc-1 to *P. aeruginosa* pathogenesis in the lung, and assuming that the significant mechanical damage that is inflicted on host tissue during thermal injury results in high levels of shed Sdc-1, we hypothesized that shed Sdc-1 is a decisive host factor contributing to *P. aeruginosa* pathogenesis in burn wound infections. Here we report that Sdc-$1^{-/-}$ mice are significantly less susceptible to *P. aeruginosa* burn wound infections than WT mice and that shed Sdc-1 may play a role in the blood vessel invasion and subsequent systemic spread of *P. aeruginosa*. These data provide support for the prospect of using Sdc-1 neutralizing agents in the treatment of burn wounds.

MATERIALS AND METHODS

Mice. Sdc-$1^{-/-}$ mice were generated and bred as previously described (22). Sdc-$1^{-/-}$ mice used in this study were backcrossed eight times onto the C57BL/6 background and were maintained by interbreeding at Baylor College of Medicine (Houston, Tex.). WT Sdc-$1^{+/+}$ C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA) or were derived from crossing mice heterozygous for the syndecan 1 gene. Mice used in experiments were 6 to 8 weeks old and weighed 17 to 20 g. Mice were housed and studied under protocols approved by the Institutional Animal Care and Use Committee in the animal facility of Texas Tech University Health Sciences Center (Lubbock, TX).

Bacterial growth and inoculum. *P. aeruginosa* strain PAO1 was grown in Luria-Bertani (LB) medium (1). Aliquots (50 μl) of overnight cultures of PAO1 were subcultured in fresh LB broth and grown at 37°C for 4 h to an optical density at 540 nm of approximately 0.9. A 100-μl aliquot of each culture was then pelleted, washed in phosphate-buffered saline (PBS), and serially diluted (10-fold serial dilutions) in PBS. A 100-μl aliquot of the 10^{-4} dilution was injected into each animal. As we have previously determined (9), this dilution contains approximately 2×10^2 to 2×10^3 CFU of *P. aeruginosa*. We have also shown previously that this dose of PAO1 produces 90 to 100% lethality in WT C57BL/6 mice by 48 h postburn infection (9). The exact inoculum of each strain was determined by plating serial dilutions of the inoculum on LB agar plates.

Thermally injured mouse model. The virulence of PAO1 was examined by using the modified burned-mouse model of Stieritz and Holder (35). In this modified model, a scald burn is induced. Mice were anesthetized by intraperitoneal injection of 0.4 ml of Nembutal/20 g of body weight at 5 mg/ml (5% sodium pentobarbital; Abbott Laboratories, North Chicago, Ill.), and their backs were shaved. The mice were securely placed into a template with an opening (4.5 by 1.8 cm) exposing their shaved backs. About 15% of the total surface area of the mouse was exposed through the opening on the template. The thermal injury was induced by placing the exposed area of the shaved skin in 90°C water for 10 s. Such an injury is nonlethal but causes a third-degree (full-thickness) burn. Fluid replacement therapy consisting of a subcutaneous injection of 0.8 ml of a 0.9% NaCl solution was administered immediately following the burn. Mice were challenged by the subcutaneous inoculation of 100 μl of the bacterial inoculum (see above) directly under the burn. Control mice were subcutaneously injected with 100 μl of sterile phosphate-buffered saline (PBS) directly under the burn. In some experiments, porcine mucosal heparan sulfate (Sigma, St. Louis, Mo.) dissolved in PBS was simultaneously injected under the burn with the bacterial inoculum. During recovery, the mice were continuously observed under warming lights.

Mortality among infected mice was recorded at 24 h intervals, up to 5 days postburn and postinfection (postburn/infection). Animals were treated humanely and in accordance with the protocol approved by the Animal Care and Use Committee at Texas Tech University Health Sciences Center (Lubbock, TX).

Quantitation of bacteria within the skin and livers. At 24 h postburn/infection, mice were euthanized by intracardial injection of 0.2 ml of Sleepaway (sodium pentobarbital-7.8% isopropyl alcohol euthanasia solution; Fort Dodge Laboratories, Inc., Fort Dodge, Iowa). Skin sections of approximately 5 by 5 mm were obtained from the burned skin of both the control and challenged mice. Simultaneously the entire liver of each animal was obtained. Tissues were weighed, suspended in 2 ml of PBS, and homogenized (overhead stirrer; Wheaton Instruments, Millville, N.J.). Homogenates were serially diluted, and a 100-μl aliquot of each diluent was plated on LB agar plates to determine the number of CFU, which was calculated per gram of tissue.

Immunoblotting experiments. Skin sections from control (nonburned) mice, mice that had been administered a third-degree scald burn, and mice that were burned and infected with *P. aeruginosa* (PAO1) were harvested at 24 h postburn. The tissue was placed in phosphate-buffered saline (PBS) and gently vortexed for 10 s. Five-microliter samples of the wound fluid extracts were then dot blotted in triplicate onto a cationic nylon membrane (Immobilon Ny+; Millipore) and quantified by dot immunoblotting with the 281-2 rat anti-mouse Sdc-1 ectodomain monoclonal antibody as described previously (21, 23). The same blot was stripped and then reprobed with an antibody directed against the cytoplasmic domain of Sdc-1 (21) to confirm that Sdc-1 detected by the 281-2 antibody was shed ectodomain and not intact Sdc-1 released from cells damaged by vortexing.

Hematoxylin and eosin (H&E) staining. Mouse skin was harvested and immediately placed into 4% paraformaldehyde in phosphate buffer, pH 7.3, and fixed overnight at 4°C. The tissue was then processed through a graded series of ethanol, cleared in xylene, and infiltrated with paraffin. The tissue was embedded on edge in paraffin, and blocks were sectioned with a Cut 4055 Olympus microtome. Five-micrometer sections of the blocks were collected on Superfrost/Plus slides (Fisher Scientific) and H&E stained using standard laboratory techniques. Slides were visualized by light microscopy at 400× with an Olympus BH-2 microscope and imaged with an Olympus camera model C-35AD-4 (Tokyo, Japan).

Scanning electron microscopy. Mouse skin that had been burned and infected with *P. aeruginosa* was harvested, cut into 1-mm-thick cross-sections, and placed into fixative containing 1.5% glutaraldehyde and 3% paraformaldehyde in 0.1 M Millonig's buffer with dextrose. Mouse skin that had been burned but not infected was treated in a similar manner to use as a control. The tissues were fixed overnight at 4°C. Tissue was then frozen in liquid nitrogen and fractured with a frozen blade to expose more surface. Tissues were then washed in buffer, post-fixed in 1% osmium tetroxide, and taken through a graded series of alcohols. The tissue was critically point dried with a Tousimis critical point dryer (Rockville, MD). The sections were mounted on edge on stubs coated with carbon tape. Some of the infected skin tissue samples were carved using a razor blade under a dissecting scope to reveal more of the inner structure. All the tissues were gold coated using a Polaron sputter coater. The sections were then scanned for bacteria, and pictures were taken using a Hitachi S-500 scanning electron microscope (Hitachi America, Ltd., Brisbane, CA).

Cytokine expression. Total RNA from mouse skin sections was extracted using TRI Reagent-RNA/DNA/Protein isolation reagent per the manufacturer's guidelines (Molecular Research Center, Inc., Cincinnati, OH). RNA was treated with RQ1 RNase-Free DNase (Promega, Madison, WI) to remove any contaminating genomic DNA. A total of 20 μg of DNA-free RNA was used to detect the expression of murine cytokine genes using the mck4 and mck3b Multi-Probe Template Sets and the Riboquant RNase Protection Assay System (PharMingen, San Diego, CA). The probes were hybridized overnight at 56°C with 20 μg of total RNA from either the liver or the skin. The mixtures were then RNase treated, purified, and resolved on a 6% acrylamide–8 M urea gel per the manufacturer's guidelines. Gels were dried, and protected bands representing different cytokines were detected after exposure of the gels to a phosphorimager screen. Radioactive signals were imaged using a Typhoon variable mode imager (Amersham Biosciences, Piscataway, NJ). Signals were quantitated

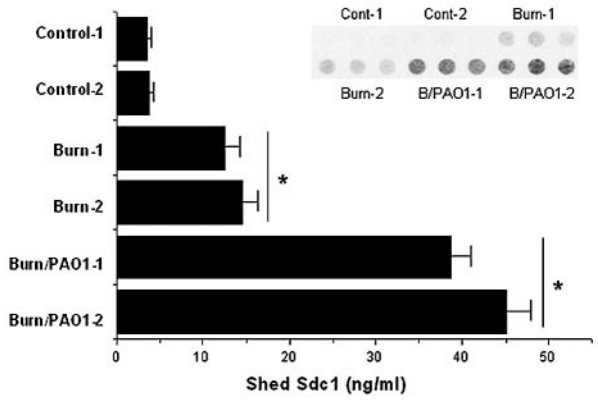

FIG. 1. Immunoblotting with Sdc-1 antibody. Skin sections from two control (nonburned) mice, two mice that had been administered a third-degree scald burn, and two mice that were burned and infected with PAO1 were placed into PBS and gently vortexed. Samples (in triplicate) from these wound fluid extracts were transferred to cationic nylon membranes and quantified by dot immunoblotting with a monoclonal antibody to Sdc-1 ectodomain. Data shown represent one experiment and are means ± standard errors of the means (*, $P < 0.01$ between combined control and burn groups and control and burn/PAO1 groups; Student's *t* test). Cont, control.

using Image-Quant TL (Amersham Biosciences, Piscataway, NJ). The signal of the band representing a cytokine of interest was normalized to the signal of the L32 (ribosomal gene) band from the same sample.

Statistical analysis. The Student's *t* test and the Fisher's exact test (Statview; Abacus Concepts, Inc., Berkeley, CA) were used to determine significant differences between the numbers of CFU/gram of tissue in bacterial colonization experiments and between groups for the mortality experiments, respectively. Significant differences between cytokine mRNA levels in the RNase protection assays were determined by the Student's *t* test (Microsoft Excel 2002, Austin, TX).

RESULTS

Thermally injured syndecan 1 knockout mice are less susceptible to *P. aeruginosa* infection. Tissue injury activates syndecan shedding (12), and shed Sdc-1 is found at high levels in human dermal wound fluids (37) and in tracheal aspirates of ventilated preterm infants (13). However, Sdc-1 has not been demonstrated in burn wounds. To determine if thermal injury results in Sdc-1 shedding, Sdc-1 ectodomain levels in burn fluid were measured by dot immunoblotting. Skin sections from control (nonburned) mice, mice that had been administered a third-degree scald burn, and mice that were burned and infected with *P. aeruginosa* (PAO1) were harvested at 24 h postburn. The tissue was placed in phosphate-buffered saline (PBS) and gently vortexed for 10 s. Samples were then dot blotted onto a cationic nylon membrane and quantified by immunoblotting with an Sdc-1 ectodomain antibody. Sdc-1 was detected in the fluid extract of burned, but not nonburned, skin; however, increased levels of Sdc-1 were detected in the wound fluid extracts of mice that were burned and infected with PAO1 (Fig. 1). Sdc-1 ectodomains were not detected when the membrane was reprobed with an antibody against the cytoplasmic domain of Sdc-1, confirming that Sdc-1 was shed and not released intact from cells (data not shown).

To determine if the increased levels of shed Sdc-1 that are associated with thermal injury correlated with the severity of *P. aeruginosa* infection, we utilized Sdc-$1^{-/-}$ mice, which carry a deletion in the gene encoding Sdc-1, and the thermally injured mouse model. Sdc-$1^{-/-}$ mice are fertile and develop normally (8). The thermally injured mouse model of *P. aeruginosa* infection was originally described by Stieritz and Holder and closely resembles the pathology of human thermal injury (35). We have previously determined that the *P. aeruginosa* strain PAO1 causes approximately 90% mortality to thermally injured mice by 48 h postburn/infection (30, 31).

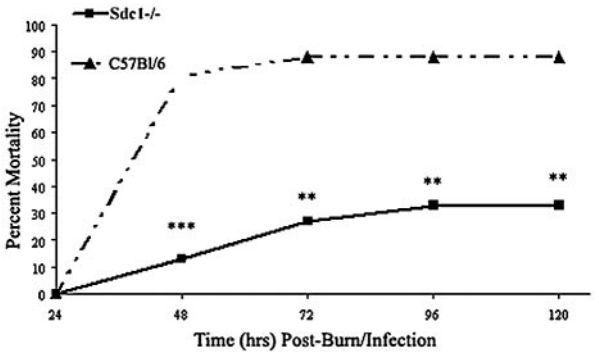

FIG. 2. Percent mortality in PAO1-infected thermally injured WT and Sdc-$1^{-/-}$ mice. Three groups of mice (5 to 8 mice per group) were thermally injured, inoculated with PAO1, allowed to recover, and monitored over 5 days (120 h). Mortality was assessed at 24-h time points. Percent mortality was significantly decreased in Sdc-$1^{-/-}$ mice compared to WT mice at all time points (**, $P < 0.005$; ***, $P < 0.001$; Fisher's exact test). Data shown represent three independent experiments.

Groups of WT or Sdc-$1^{-/-}$ mice on the C57BL/6 background were given full-thickness scald burns and infected with PAO1. The mice were observed for 120 h (5 days) postburn/infection, and percent mortality was recorded for each group (Fig. 2). Eighty-two percent (18 out of 22) of the burned and infected C57Bl/6 WT mice died in the first 48 h, and no further mortality was observed. However, the percent mortality of Sdc-$1^{-/-}$ mice was significantly reduced at all time points (Fig. 2; 13% [2 out of 15] at 48 h [$P = 0.00005$] and 40% [6 out of 15] at 120 [$P = 0.014$]).

Shed Sdc-1, via its HS chains, can function as a soluble effector to regulate various cellular processes, including proliferation, adhesion, and differentiation (3). In a *P. aeruginosa* lung infection model, HS was as effective as purified shed Sdc-1 at increasing the susceptibility of Sdc-$1^{-/-}$ mice to lung infection from *P. aeruginosa* (22). To determine if the absence of shed Sdc-1 was responsible for the lower susceptibility of burned Sdc-$1^{-/-}$ mice to *P. aeruginosa* infection, we tested whether HS could increase mortality. Sdc-$1^{-/-}$ mice were burned and infected with PAO1 as before; however, some groups of mice were also administered a single 20-μg, 50-μg, or 100-μg dose of HS locally at the injury site. These doses were chosen because we have previously determined that 100 μg of protamine sulfate is required to neutralize syndecan 1 in vivo (9). As before, the percent mortality at 48 h in Sdc-$1^{-/-}$ mice infected with PAO1 was relatively low 25% (2 out of 8 mice). However, the percent mortality was significantly higher (83%, 5 out of 6 mice; $P = 0.05$; for 100 μg) in mice that received a simultaneous HS injection and increased to 100% by the 72-h time point (Fig. 3). The percent mortality among HS-injected

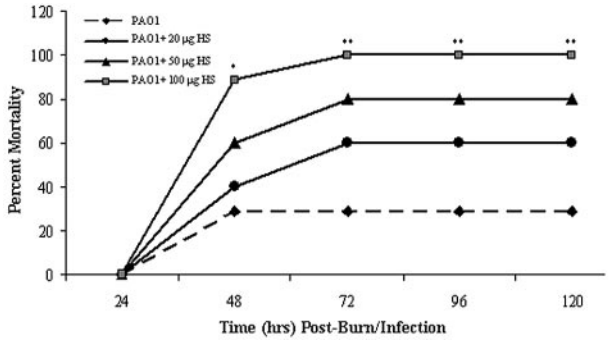

FIG. 3. Heparan sulfate increases percent mortality in Sdc-1$^{-/-}$ mice. Two groups of Sdc-1$^{-/-}$ mice were thermally injured and inoculated with PAO1. Mice were injected with a single dose of 20, 50, or 100 μg HS, and a control group received PBS (3 to 4 mice per treatment). The mice were then monitored for 5 days for mortality (*, $P < 0.05$; **, $P < 0.01$; Fisher's exact test). Data shown represent two independent experiments.

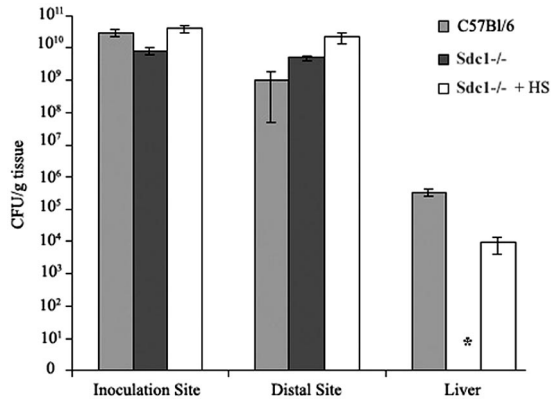

FIG. 4. Colonization and spread of PAO1 in thermally injured WT and Sdc-1$^{-/-}$ mice. WT and Sdc-1$^{-/-}$ mice were anesthetized, thermally injured, and inoculated with PAO1. One group of Sdc-1$^{-/-}$ mice was also given 100 μg HS. Mice were sacrificed at 24 h postburn/infection. The livers and sections of thermally injured tissue from two sites (the bacterial inoculation site and a site 15 mm distal) were taken from each mouse and homogenized. The samples were diluted and plated on selective media to determine the number of PAO1 microorganisms (CFU/gram of tissue). Values represent the average of 7 mice/group ± standard errors of the means (*, $P < 0.01$; Student's *t* test). Data shown represent three independent experiments.

mice was similar to that seen for WT mice that possess Sdc-1 (compare Fig. 2 to 3). No mortality was observed in nonburned mice that were given 100 μg HS or infected with PAO1 plus 100 μg HS (data not shown). Taken together, these results indicate that the high levels of Sdc-1 shed due to thermal injury exacerbate the virulence of *P. aeruginosa*.

Shed syndecan 1 facilitates the systemic spread of *P. aeruginosa*. *P. aeruginosa* infection in the murine thermal injury model can be divided into two phases (10, 11). The first phase involves colonization of the burned tissue by *P. aeruginosa*. The devascularized state of the burn eschar (burn wound area) provides an environment ideally suited for bacterial growth, and *P. aeruginosa* proliferates extremely quickly. Once a threshold concentration of bacteria is reached in the eschar (approximately 10^9 CFU/g tissue), *P. aeruginosa* spreads systemically, causing sepsis followed by multiple-organ failure and eventually death (10, 11). We have previously seen that by 24 h postburn/infection, *P. aeruginosa* CFU in the eschar increase from 10^2 CFU/g tissue (infecting dose) to approximately 10^9 CFU/g tissue and can also be isolated from the liver and spleen (29, 32). By 48 h postburn/infection, the majority of mice succumb to the infection (29, 32).

To determine if Sdc-1 influenced either stage of *P. aeruginosa* infection, we examined both the local colonization within the eschar and the systemic spread of *P. aeruginosa* in burned and infected WT and Sdc-1$^{-/-}$ mice. We examined the local colonization of bacteria within the eschar by determining the number of PAO1 CFU in epithelial sections taken at two specific sites within the area of thermal injury, the inoculation site and a site 15 mm distal to the inoculation site. Using this approach, we have previously shown that PAO1 spreads efficiently from the inoculation site to the distal site by 24 h postburn (32). As in the mortality experiments, mice were thermally injured and inoculated with PAO1. At 24 h postburn/infection, the mice were euthanized. Epithelial sections were isolated and homogenized, and the number of PAO1 CFU was determined as described in Materials and Methods. WT and Sdc-1$^{-/-}$ mice had similar *P. aeruginosa* CFU counts within both the "inoculation" and "distal" epithelial sections (Fig. 4), and administration of HS did not affect local spread. These results indicate that within 24 h, *P. aeruginosa* is able to efficiently multiply and colonize the burn eschar despite the lack of Sdc-1.

To examine the systemic spread of *P. aeruginosa*, mice were thermally injured and inoculated with PAO1 as described above. At 24 h postinfection, the mice were euthanized, the livers were harvested, and the CFU/gram of tissue was determined. On average, $3.4 \times 10^5 \pm 9 \times 10^4$ CFU were recovered from livers of WT mice (5 mice/group), while no bacteria were recovered from livers of Sdc-1$^{-/-}$ mice (5 mice/group). Administration of heparan sulfate, as described above, resulted in similar CFU counts as seen in WT mice ($9 \times 10^3 \pm 5 \times 10^3$). Taken together, these results indicate that *P. aeruginosa* can efficiently colonize the burn eschar and multiply to threshold levels despite the absence of Sdc-1. However, Sdc-1 appears to be needed for dissemination of PAO1 into the bloodstream and secondary colonization of internal organs.

Syndecan 1 is involved in *P. aeruginosa* perivascular cuffing. Two distinctive clinical features of *P. aeruginosa* bacteremia are invasion and necrosis of blood vessels (34). In humans, blood vessel invasion by *P. aeruginosa* correlates with the appearance of skin lesions termed ecthyma gangrenosum (17). Ecthyma gangrenosum is classically considered a pathognomonic sign of sepsis by *P. aeruginosa* (17, 18). However, a few other bacterial species have now been associated with ecthyma gangrenosum (27). Upon microscopic examination, blood vessel invasion by *P. aeruginosa* can be recognized by the presence of perivascular cuffing (PVC) (34). Bacilli form a circumferential pattern surrounding the vessel and are often aligned single file or in stacks between muscle cells of the venous walls (34). The mechanisms of PVC by *P. aeruginosa* and its role in pathogenesis are not fully understood. However, in the thermally injured mouse model, we have consistently seen that PVC accompanies sepsis and

subsequent death from *P. aeruginosa* infection (K. P. Rumbaugh, unpublished observations).

As Sdc-1 is a component of normal vascular endothelium and Sdc-1$^{-/-}$ mice displayed significantly less sepsis from *P. aeruginosa* infection, we wanted to determine if Sdc-1 played a role in PVC. Groups of mice were thermally injured and infected with PAO1 as described in Materials and Methods. At 24 h postburn/infection, mice were euthanized and skin sections were harvested for histological evaluation. Sections measuring approximately 1 by 1.5 cm were extracted from the burn margin (the area where burned and healthy tissue merge). PVC was visualized in hematoxylin and eosin (H&E)-stained sections by the appearance of circumferentially arranged gram-negative bacilli around one or more blood vessels (Fig. 5A). PVC was usually seen in association with vessel thrombosis or blood clotting within the vessel, as seen in Fig. 5A. PVC was observed in skin sections from 4 of 5 thermally injured, *P. aeruginosa*-infected WT mice. However, none of the five sections obtained from Sdc-1$^{-/-}$ mice displayed PVC. Although bacilli could be seen in tissue sections from Sdc-1$^{-/-}$ mice, they were dispersed throughout the section and the blood vessels appeared normal without thrombosis (Fig. 5B). Sdc-1$^{-/-}$ mice that were administered HS in addition to PAO1 also displayed extensive PVC (data not shown). Skin tissue from thermally injured WT mice infected with PAO1 was also visualized by electron microscopy. Scanning electron microscopy (SEM) revealed organized arrangements of *P. aeruginosa* bacilli in areas surrounding blood vessels (Fig. 5C).

Syndecan 1 modulates the host cytokine response to *P. aeruginosa* infection in thermally injured mice. Extensive inflammation is an important initiating event in thermal injury that predisposes the host to sepsis, multiorgan failure, and death (6, 40). We have previously shown that the combination of thermal injury and *P. aeruginosa* infection results in an acute and exaggerated inflammatory response that is characterized by elevated mRNA levels of several cytokine and chemokine genes (29). This "cytokine storm" can be detected locally in the skin and systemically within the livers of burned and PAO1-infected mice by 24 h postburn/infection (29). Shed Sdc-1 is known to regulate the expression and function of many cytokines (7). Sdc-1 stimulates the release of TNF-α, IL-1β, and IL-6 from dendritic cells and IL-1, IL-6, TNF-α, IL-12, transforming growth factor-β (TGF-β), and prostaglandin E2 (PGE2) from macrophages (7), and it is known to play a key role in IL-8-mediated chemotaxis of neutrophils to sites of tissue injury (15). As Sdc-1 shedding on epithelial cells accompanies thermal tissue injury, we wanted to determine if Sdc-1 was a mediator in the extreme upregulation of cytokines seen at the onset of thermal injury. Therefore, we examined the mRNA levels of 19 murine cytokine and chemokine genes in both the skin and livers of thermally injured, PAO1-infected WT or Sdc-1$^{-/-}$ mice. The murine genes examined were TNF-α, TNF-β, IL-3, IL-6, IL-7, IL-11, TGF-β1, TGF-β2, TGF-β3, gamma interferon (IFN-γ) and IFN-β, lymphotoxin β (LTβ), macrophage migration inhibitory factor (MIF), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF), leukocyte inhibitory factor (LIF), and stem cell factor (SCF).

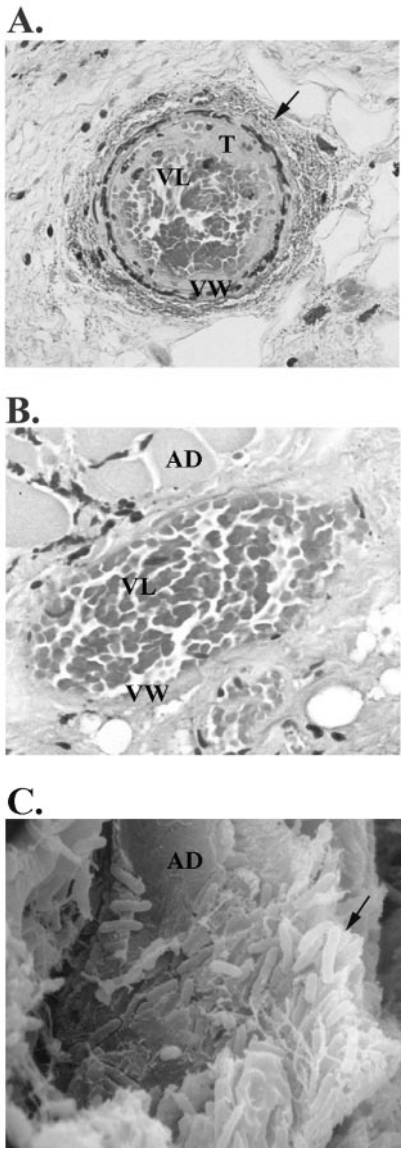

FIG. 5. Visualization of PVC of *P. aeruginosa*-infected skin sections. WT and Sdc-1$^{-/-}$ mice were thermally injured and inoculated with PAO1. At 24 h postburn/infection, mice were sacrificed and skin sections were harvested for H&E staining or electron microscopy. Sections measuring approximately 1 by 1.5 cm were extracted from the burn margin (the area where burn and healthy tissues merge). PVC were visualized at 400× in H&E-stained sections from WT mice by the appearance of circumferentially arranged gram-negative bacilli (indicated by arrow) around one or more blood vessels (A). Vessels in skin sections from Sdc-1$^{-/-}$ mice appeared normal (B), with no PVC or thrombosis. AD, VW, VL, and T indicate adipocyte, vessel wall, vessel lumen and thrombosis, respectively. Areas of the connective tissue adjacent to blood vessels were examined by scanning electron microscopy (SEM). *P. aeruginosa* bacilli (indicated by arrow) were visualized by SEM at 7,000× in sheeted layers (C) between the adipose (AD) and muscle cells surrounding vessels. These images are representative of mouse tissue visualized from at least three independent experiments.

WT and Sdc-1$^{-/-}$ mice were thermally injured and infected with PAO1. Mice were euthanized at 24 h postburn/infection, and RNA was harvested from skin and liver sections as described in Materials and Methods. RNase protection assays

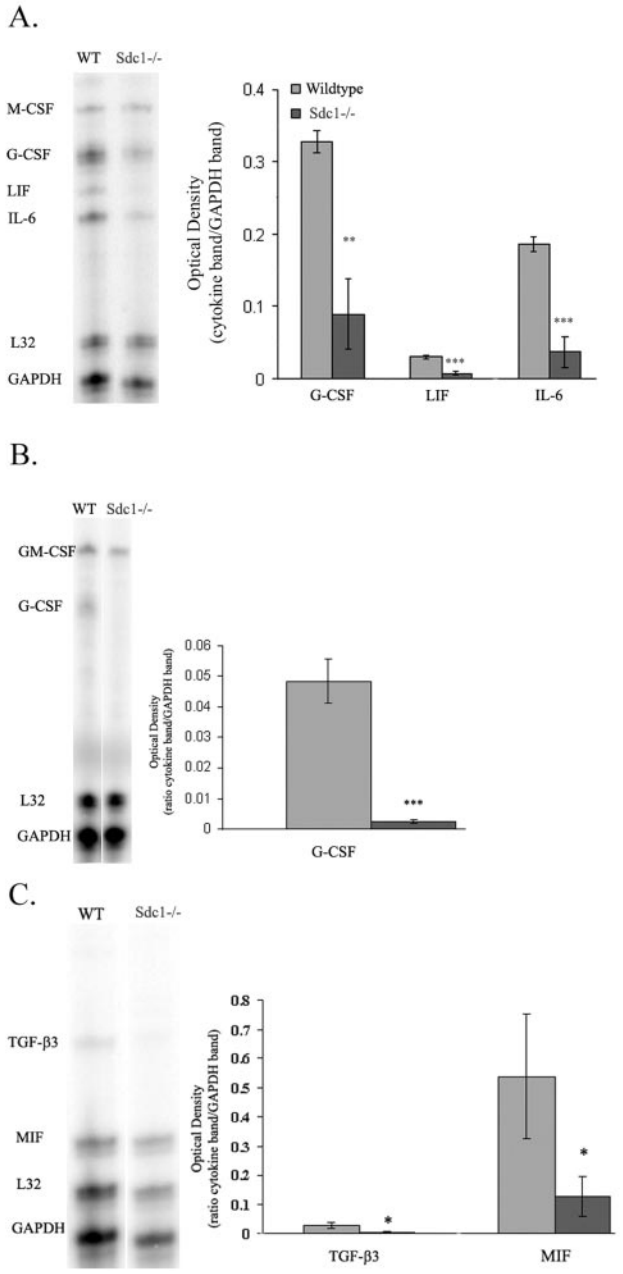

FIG. 6. Cytokine response to PAO1 infection in thermally injured C57Bl WT and Sdc-1$^{-/-}$ mice. Mice were burned and infected with PAO1. At 24 h postburn/infection, they were sacrificed and tissue samples were harvested. RNA was isolated from viable skin tissue surrounding the burn margin (A) and from liver (B and C). Twenty micrograms of DNased RNA was used for RPA using the mck4 (A and B) or mck3b (C) template (BD RiboQuant; BD Biosciences) to examine the mRNA levels representing several cytokines. Representative RPA are shown. The results of group data ($n = 4$ mice/group) from two separate experiments were expressed as the mRNA level of the cytokine of interest normalized to that of L32 (ribosomal gene) from the same sample ± standard deviations (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; Student's t test).

(RPA) were run using the Multi-Probe RPA System (BD RiboQuant). Transcripts for many of the cytokines and chemokines examined were detected in the skin of both WT and Sdc-1$^{-/-}$ mice. As we have previously reported (29), MIF, M-CSF, TGF-β, TNF-α, IL-6, IFN-γ, IFN-β, LIF, and G-CSF were all expressed in thermally injured, PAO1-infected skin (data not shown). However, the mRNA levels of G-CSF, LIF, and IL-6 were significantly decreased in the skin of Sdc-1$^{-/-}$ mice (Fig. 6A). In this study, and in agreement with our previous studies (29), the only cytokines from those examined that were detected in the livers of burned and infected mice were MIF, TGF-β, G-CSF, and GM-CSF. However, Sdc-1$^{-/-}$ mice displayed significantly lower levels of MIF, TGF-β, and G-CSF (Fig. 6B and C). Taken together, these data indicate that despite the absence of Sdc-1, Sdc-1$^{-/-}$ mice display a significant local inflammatory cytokine response; however, some of the cytokines that are characteristic of PAO1 infection are reduced. Conversely, the systemic inflammatory response, as illustrated by cytokine expression in the livers, was almost completely absent in Sdc-1$^{-/-}$ mice (Fig. 6B and C). This is consistent with the lack of systemic spread observed in Sdc-1$^{-/-}$ mice.

DISCUSSION

Serious thermal injuries result in dysregulation of immune responses and a hypermetabolic state that can cause multiple-organ failure and predispose patients to bacterial infections (5). *P. aeruginosa* is one of the most common and pathogenic bacteria encountered in burn wound infections (26, 39). *P. aeruginosa* can rapidly colonize burn wounds, resulting in extensive inflammation, bacteremia, and high mortality. As thermal injury causes a complicated clinical scenario, it is likely that there are many host and bacterial factors that contribute to *P. aeruginosa* pathogenesis in burn wounds. The goal of this study was to examine the contribution of one such host factor, Sdc-1, in *P. aeruginosa* infection of burn wounds. Taken together, our data indicate that Sdc-1 increases the susceptibility of thermally injured mice to *P. aeruginosa* infection. We show that Sdc-1$^{-/-}$ mice display reduced inflammatory cytokine expression, sepsis, and mortality upon a *P. aeruginosa* infection of a burn wound in comparison to WT mice.

Tissue injury activates shedding of Sdc-1, and high levels of shed Sdc-1 have been documented in human dermal wound fluids (37). In this study we demonstrate for the first time in vivo that Sdc-1 is shed by the trauma of thermal injury, and shedding is increased further by *P. aeruginosa* infection (Fig. 1). Although the LasA protease produced by *P. aeruginosa* can promote Sdc-1 shedding (23), we did not observe a reduction in mortality in thermally injured mice infected with a PAO1::*lasA* isogenic mutant (unpublished data). This indicates to us that the levels of shed Sdc-1, which are already increased due to the thermal injury, are not enhanced by the action of LasA. However, it is possible that in a *P. aeruginosa* lung infection where there are no reservoirs of shed Sdc-1, a LasA mutant would display reduced virulence. Consistent with this hypothesis, we detected >10 ng/ml of Sdc-1 ectodomains in tissues that were gently vortexed for 10 s, suggesting that a significantly larger concentration of Sdc-1 is present in thermally injured tissue.

Our results are consistent with previous observations suggesting that *P. aeruginosa* exploits shed Sdc-1 to enhance its virulence in the lung (22). Similar to data reported by Park et al., we see a significant decrease in mortality of *P. aeruginosa*-infected, thermally injured Sdc-1$^{-/-}$ mice, and this reduction can be compensated for by the addition of HS. Park et al. reported that *P.*

aeruginosa was reduced in the primary (lung) and secondary (spleen and liver) infection sites in their pneumonia model (22). However, in the thermally injured mouse, we observed that *P. aeruginosa* was not inhibited from efficiently colonizing the primary infection site (burn wound) but could not spread systemically. It is possible that this inconsistency is due to the devascularized state of the burn wound that allows an uncontrolled proliferation of *P. aeruginosa*. The infected lung maintains a sufficient blood supply that provides access for immune system mediators to the area. It is possible that more subtle contributions of Sdc-1 to the actions of these mediators are more easily observed in the lung infection model. Despite differences in the local colonization of *P. aeruginosa*, both studies observed significant decreases in the systemic spread of *P. aeruginosa* in Sdc-1$^{-/-}$ mice (this study and reference 22). Furthermore, in both studies systemic spread was returned to WT levels by the addition of HS. Taken together, these observations provide strong evidence that the mechanism by which shed Sdc-1 enhances *P. aeruginosa* pathogenesis is related to the ability of *P. aeruginosa* to disseminate and cause sepsis.

We observed prevalent PVC of *P. aeruginosa* in skin sections from WT mice, but no PVC was seen in sections from Sdc-1$^{-/-}$ mice (Fig. 5A and B). As PVC is generally accompanied by blood vessel invasion (17, 34), it is possible that Sdc-1 shedding facilitates blood vessel invasion in thermally injured mice. This possibility is consistent with the lack of *P. aeruginosa* systemic spread in Sdc-1$^{-/-}$ mice and could explain the reduction in mortality. However, the mechanisms by which shed Sdc-1 enhances blood vessel invasion are unknown at this time. One possible explanation is that the transmembrane core protein that remains in the vascular endothelial cell membrane after shedding acts as an attachment site for *P. aeruginosa*. Some bacteria, including *P. aeruginosa*, use HSPGs for adherence and invasion; however, these interactions typically rely on bacterial adherence to the anionic HS side chains and not to the core protein (24, 28). Furthermore, Park et al. determined that *P. aeruginosa* did not bind to ^{125}I- or ^{25}S-labeled Sdc-1 ectodomains (22). Interestingly, the phenomenon of PVC is almost exclusive to the *Pseudomonas* and *Aeromonas* species (17) and has been relatively understudied. The correlation of PVC with sepsis and mortality implicates it as an important mechanism for *P. aeruginosa* pathogenesis, especially in wounds. The striking absence of PVC in Sdc-1$^{-/-}$ mice leads us to propose that there are important interactions between *P. aeruginosa* and Sdc-1 itself or an Sdc-1-regulated factor on vascular endothelial cells that deserve further investigation.

Upon thermal injury and *P. aeruginosa* infection, we detected significant reductions in the mRNA levels for G-CSF, LIF, and IL-6 in the dermal tissue and G-CSF, TGF-β3, and MIF in the livers of Sdc-1$^{-/-}$ mice in comparison to WT mice (Fig. 6). However, despite these reductions, Sdc-1$^{-/-}$ mice displayed a significant cytokine response to the thermal injury and subsequent *P. aeruginosa* infection. Most of the 19 murine cytokine and chemokine genes we examined were detected in both WT and Sdc-1$^{-/-}$ mice, including the important proinflammatory cytokines IFN-γ and TNF-α. The cytokine response elicited in thermally injured Sdc-1$^{-/-}$ mice by *P. aeruginosa* may help explain why some mice succumbed to the infection in the absence of systemic spread of *P. aeruginosa.* High levels of circulating cytokines are associated with poor clinical outcomes in trauma patients (4). Circulating proinflammatory cytokines can cause dysfunction of the renal, cardiovascular, respiratory, nervous, and musculoskeletal systems, resulting in systemic inflammatory response syndrome and eventual multiple-organ failure (4).

Significant *P. aeruginosa* CFU were detected in the livers of WT but not Sdc-1 mice (Fig. 4). Therefore, the most likely explanation for the difference in the systemic inflammatory response to PAO1 in WT versus Sdc-1$^{-/-}$ mice is the absence of significant numbers of bacteria within the livers of Sdc-1$^{-/-}$ mice. However, the differences in the local inflammatory response are less apparent. Similar numbers of *P. aeruginosa* were detected in skin sections from WT and Sdc-1$^{-/-}$ mice (Fig. 4), therefore the reductions in IL-6, LIF, and G-CSF message are not likely to be due to *P. aeruginosa*-derived factors. Sdc-1 is known to induce production of IL-6 from dendritic cells and to induce IL-6 and TGF-β from macrophages (7). However, in our model, acute inflammation is observed within a few hours following burn and infection, sepsis occurs in the first 24 h, and death is noted by 48 h (29, 33). Macrophages and dendritic cells, which are not usually detected at high numbers in burned tissue for several days following thermal injury, are less likely to be responsible for local cytokine production (16). While many types of cells produce these cytokines, likely sources in our model include activated neutrophils, fibroblasts, and/or endothelial cells within the dermal tissue and hepatocytes within the liver. Sdc-1$^{-/-}$ mice display increased vascular permeability and neutrophil extravasation (8). Therefore, we attempted to determine if the neutrophil response of Sdc-1$^{-/-}$ mice to *P. aeruginosa* was more prominent than that in WT mice. In order to investigate this possibility, we examined skin sections from WT and Sdc-1$^{-/-}$ mice burned and infected with *P. aeruginosa*. We did not detect differences in the numbers of neutrophils present upon visual examination of H&E-stained sections or sections immunostained with anti-GR-1 (differentiation marker for mouse neutrophils). Sections from both WT and Sdc-1$^{-/-}$ burn wounds displayed a large neutrophil infiltrate (data not shown). In order to determine if the neutrophils present within the tissue were activated, we performed immunohistochemistry with antibody to CD11b, a cell surface antigen present on activated neutrophils. No differences in staining patterns were observed between WT and Sdc-1$^{-/-}$ mice (data not shown). Therefore, both WT and Sdc-1$^{-/-}$ mice elicit a substantial neutrophil response to thermal injury and *P. aeruginosa* infection. Following extravasation into infected tissue, activated neutrophils release oxidants and hydrolytic enzymes that are intended to aid in eradication of infecting bacteria (36). However, shed Sdc-1 has been shown to bind the neutrophil serine proteases elastase and cathepsin G (12). Therefore, it is possible that shed Sdc-1 binds and inhibits the activity of one or more neutrophil-derived antimicrobial products, thereby negating the protective effects of activated neutrophils. However, this possibility is less likely, considering that we see no difference in local colonization of *P. aeruginosa* in WT versus Sdc-1$^{-/-}$ mice.

Our data as well as data from other investigators indicate that Sdc-1 shedding is likely to play an important role in the innate immune response following wound and lung infections. *Staphylococcus aureus*, another important opportunistic pathogen, can also promote Sdc-1 shedding through the action of alpha and beta toxins (21). Interestingly, *P. aeruginosa* and *S. aureus* are two of the major pathogens responsible for both lung and skin wound infections (22). Another commonality of these two pathogens is

their increasing antibiotic resistance. Two recent studies determined that 14% of *P. aeruginosa* (38) and 56% of *S. aureus* (20) bloodstream infections were caused by multidrug-resistant strains. Therefore, the identification of Sdc-1 shedding as a common mechanism of pathogenesis could be exploited to develop alternative treatments for both *P. aeruginosa* and *S. aureus* infections, which often occur simultaneously. We have recently shown that treatment with protamine sulfate, which neutralizes the activity moiety of Sdc-1 (HS), had beneficial effects on the survival of thermally injured *P. aeruginosa*-infected mice (9). In fact, thermally injured, *P. aeruginosa*-infected WT mice that were treated with protamine sulfate displayed mortality levels consistent with those seen for Sdc-$1^{-/-}$ mice in this study. Protamine-treated mice also displayed significantly lower levels of sepsis and proinflammatory cytokine expression, also consistent with our observations in Sdc-$1^{-/-}$ mice. Protamine sulfate has also successfully been used in treatment of *P. aeruginosa* infection in a mouse pneumonia model (22). It will be extremely interesting to know if protamine sulfate is effective in inhibiting the pathogenesis of *S. aureus* in lung and/or burn wound infections. A therapeutic agent effective against two major multidrug-resistant pathogens that targets a component of the host response and not the bacteria itself could be a very valuable clinical tool.

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