

RESEARCH ARTICLE

Loss of endothelial sulfatase-1 after experimental sepsis attenuates subsequent pulmonary inflammatory responses

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Oshima K, Han X, Ouyang Y, El Masri R, Yang Y, Haeger SM, McMurtry SA, Lane TC, Davizon-Castillo P, Zhang F, Yue X, Vivès RR, Linhardt RJ, Schmidt EP. Loss of endothelial sulfatase-1 after experimental sepsis attenuates subsequent pulmonary inflammatory responses. *Am J Physiol Lung Cell Mol Physiol* 317: L667–L677, 2019. First published August 28, 2019; doi:10.1152/ajplung.00175.2019.—Sepsis patients are at increased risk for hospital-acquired pulmonary infections, potentially due to postseptic immunosuppression known as the compensatory anti-inflammatory response syndrome (CARS). CARS has been attributed to leukocyte dysfunction, with an unclear role for endothelial cells. The pulmonary circulation is lined by an endothelial glycocalyx, a heparan sulfate-rich layer essential to pulmonary homeostasis. Heparan sulfate degradation occurs early in sepsis, leading to lung injury. Endothelial synthesis of new heparan sulfates subsequently allows for glycocalyx reconstitution and endothelial recovery. We hypothesized that remodeling of the reconstituted endothelial glycocalyx, mediated by alterations in the endothelial machinery responsible for heparan sulfate synthesis, contributes to CARS. Seventy-two hours after experimental sepsis, coincident with glycocalyx reconstitution, mice demonstrated impaired neutrophil and protein influx in response to intratracheal lipopolysaccharide (LPS). The postseptic reconstituted glycocalyx was structurally remodeled, with enrichment of heparan sulfate disaccharides sulfated at the 6-*O* position of glucosamine. Increased 6-*O*-sulfation coincided with loss of endothelial sulfatase-1 (Sulf-1), an enzyme that specifically removes 6-*O*-sulfates from heparan sulfate. Intravenous administration of Sulf-1 to postseptic mice restored the pulmonary response to LPS, suggesting that loss of Sulf-1 was necessary for postseptic suppression of pulmonary inflammation. Endothelial-specific knockout mice demonstrated that loss of Sulf-1 was not sufficient to induce immunosuppression in non-septic mice. Knockdown of Sulf-1 in human pulmonary microvascular endothelial cells resulted in downregulation of the adhesion molecule ICAM-1. Taken together, our study indicates that loss of endothelial Sulf-1 is necessary for postseptic suppression of pulmonary inflammation, representing a novel endothelial contributor to CARS.

compensatory anti-inflammatory response syndrome; glycomics; heparan sulfate; sulfatase-1

INTRODUCTION

Sepsis, defined as life-threatening organ dysfunction caused by a dysregulated host immune response (34), is a leading cause of in-hospital mortality worldwide (12). Classically, septic organ injury has been attributed to systemic, overwhelming hyperinflammation. However, the failures of numerous clinical trials targeting inflammatory signaling (18) led to the proposed concept that sepsis is not simply hyperinflammation but also consists of a delayed period of immunosuppression, known as the compensatory anti-inflammatory response syndrome (CARS) (1). This period of suppressed inflammation is paradoxically harmful in sepsis, imparting an increased risk for secondary infections (9, 15, 26, 35) such as hospital-acquired pneumonia (15, 39). Evidence supporting the pathologic significance of postseptic CARS includes known associations between mortality and increased plasma IL-10 and decreased human leukocyte antigen human leukocyte antigen (HLA)-DR expression on leukocytes (10, 16, 21); decreased production of cytokines, such as TNF α , IFN- γ , IL-6, and IL-10 in septic patients; and depletion of CD4+, CD8+, and HLA-DR+ cells in the spleen (2). CARS has therefore been largely attributed to dysfunctional monocytes, including impaired cytokine production and decreased phagocytosis and migration in response to inflammatory stimuli (23, 44). Despite the potential importance of CARS, clinical trials targeting immunosuppression have been disappointing (18, 19, 25), perhaps reflecting an incomplete understanding of the mechanisms responsible for postseptic impairment in lung inflammation.

The endothelial glycocalyx is a carbohydrate-rich endovascular layer that serves multiple homeostatic functions at the endothelial surface. The major glycosaminoglycan constituent of the glycocalyx is heparan sulfate (HS), a linear polysaccharide composed of repeating glucosamine and hexuronic (glucu-

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ronic and iduronic) acid disaccharide units. This disaccharide unit may be sulfated at the amino (*N*) and/or 6-*O* positions of glucosamine and/or the 2-*O* position of hexuronic acid. The resultant pattern of negative charge enables HS to bind to various cationic ligands and their cognate receptors (11, 28), influencing multiple signaling processes responsible for organ injury and repair.

We have previously reported that sepsis-induced degradation of HS from the pulmonary endothelial glycocalyx mediates alveolar neutrophil adhesion and inflammatory lung injury (31). During sepsis recovery [72 h after cecal ligation and puncture (CLP) in mice], endothelial synthesis of new HS allows for glycocalyx reconstitution, mediating endothelial recovery (45). We postulated that postseptic changes in the endothelial machinery responsible for endothelial HS synthesis lead to remodeling of the endothelial glycocalyx, impairing pulmonary responses to subsequent inflammatory stimuli.

In this report, we observed that mice demonstrated suppressed pulmonary inflammation in response to intratracheal lipopolysaccharides (LPS) after experimental sepsis, coincident with glycocalyx enrichment in 6-*O*-sulfated HS, a sulfation pattern implicated in endothelial inflammation (27, 41). This remodeling was associated with downregulation of pulmonary endothelial sulfatase-1 (Sulf-1), an enzyme responsible for the constitutive cleavage of extracellular 6-*O*-sulfo groups. We observed that loss of Sulf-1 in septic mice was necessary for the impaired pulmonary response to LPS characteristic of CARS, but it was not sufficient to cause impaired inflammation in nonseptic animals. Knockdown of Sulf-1 using siRNA in pulmonary microvascular endothelial cells resulted in downregulation of ICAM-1 transcription. Our study therefore identifies postseptic remodeling of the pulmonary endothelial glycocalyx as a novel contributor to CARS.

MATERIALS AND METHODS

Materials. We purchased LPS (from *Escherichia coli* O55:B5) and heparinase I and III (from *Flavobacterium heparinum*) from Sigma-Aldrich (St. Louis, MO) and reconstituted in phosphate-buffered (PBS). As controls, we heat-inactivated heparinase I and III at 100°C for 20 min. We purchased protein assay dye reagent from Bio-Rad (Hercules, CA). We purchased tamoxifen from Sigma-Aldrich and dissolved it in corn oil with 5% ethanol. For tissue digestion and fluorescence-activated cell (FAC) sorting, we purchased collagenase type 2 from Worthington Biochemical (Lakewood, NJ), dispase I from Sigma-Aldrich, and ACK lysing buffer from Gibco (Dublin, Ireland). We purchased anti-mouse CD31-APC antibody (17-0311-82, Clone 390) from eBioscience (San Diego, CA), anti-mouse CD144-PE antibody (562243, Clone 11D4.1) from BD Biosciences (San Jose, CA), and DAPI from Invitrogen (Carlsbad, CA). For quantitative reverse transcription-polymerase chain reaction (qRT-PCR), we purchased reagents for RNA extraction and DNase I from Qiagen (Hilden, Germany). We purchased primary human pulmonary microvascular endothelial cells from PromoCell (Heidelberg, Germany). We purchased iScript cDNA synthesis kit from Bio-Rad. We purchased siRNA targeted for sulfatase-1 (Sulf-1) from Dharmacon (Lafayette, CO) and purchased the transfection reagent Lipofectamine RNAiMAX from Thermo Fisher Scientific (Waltham, MA). For production of recombinant heparin sulfatase-1 (HSulf-1), we purchased all tissue culture reagents and media from Thermo Fisher Scientific and chromatography reagents from GE Healthcare (Chicago, IL).

Animals. All experimental protocols were approved by the University of Colorado Institutional Animal Care Use Committee, and all

experiments were performed in accordance with National Institutes of Health guidelines. Wild-type, male C57BL/6J mice (8–10 wk) were purchased from Jackson Laboratory. Sulf-1 and -2 double-floxed mice (both males and females, 8–12 wk old) were used as previously described (37). VE-cadherin CRE-ERT2 mice were provided by Dr. Ralf Adams at Max Planck Institute, Germany. Sulf-1/2 double homozygous mice with or without VE-cadherin-CRE-ERT2 (Sulf-1^{fl/fl} Sulf-2^{fl/fl} VEcadCreERT2^{- or +}) were used for all knockout animal experiments. CRE-ERT2 translocation to nucleus was induced with intraperitoneal injections of tamoxifen (dissolved in corn oil and 5% ethanol, sterile filtered), 1 mg/day, for 5 consecutive days.

Induction of sepsis in mice. We induced sepsis in 8- to 14-wk-old mice with CLP as previously described (31). Briefly, we anesthetized animals with 5% isoflurane inhalation. An incision of ~1 cm was made in abdomen, and the cecum was exposed. We ligated the cecum at 50% of its length and punctured it through and through with a 22-gauge needle. The cecum was then returned to the abdominal cavity, and the incision was closed with suture. Buprenorphine (1.6 µg/g body wt, ip) was given to each animal for pain management, and 1 mL of sterile saline was given subcutaneously as fluid resuscitation. Sham surgery was performed similarly, albeit without ligation and puncture of the cecum.

Intratracheal instillation. We anesthetized animals with 5% isoflurane inhalation, and we instilled LPS at 3 µg/g body weight to trachea visualized with laryngoscope. Animals spontaneously breathed throughout the instillation.

Blood analysis. We collected blood from the retro-orbital sinus using heparin-coated capillary tubes and determined complete blood counts using a veterinary hematology analyzer (Heska, Loveland, CO). We collected blood anticoagulated with 3.8% citrate by cardiac puncture and assessed global hemostasis using kaolin as activator by thromboelastography (Hemonetics, Braintree, MA).

Tissue and sample collection. We anesthetized mice with a lethal dose of ketamine-xylazine and collected blood in EDTA tubes by inferior vena cava cannulation. We collected plasma by centrifuging whole blood at 1,000 g for 10 min and stored it at -80°C until analysis. We cannulated the trachea and performed bronchoalveolar lavage (BAL) three times with 1 mL of PBS each. We flushed blood out of the lung by pulmonary artery perfusion, and the right lung was snap frozen with liquid N₂ and stored at -80°C until analysis. We inflated the left lung with 1% low-melting agarose in PBS, fixed with 10% formalin overnight, and processed the tissue for histological analysis. We determined total cells per milliliter in BAL fluid using a hemocytometer. We centrifuged BAL fluid at 1,200 rpm for 5 min and stored the supernatant at -80°C until analysis. We determined total protein concentration in BAL fluid with a Coomassie brilliant blue-based colorimetric assay. Cells in BAL fluid were mounted on a slide with cytospin at 600 rpm for 2 min and stained with Wright-Giemsa method for differential cell counting.

Isolated perfused mouse lung. We performed the isolated, perfused mouse lung as previously described (29). Briefly, we deeply anesthetized mice with ketamine and xylazine. After confirming the loss of toe-pinch reflex, we cannulated the trachea and ventilated mice with 21% O₂ and 5% CO₂ at 125 breaths/min at tidal volume of 250 µL. We rapidly removed the sternum and anterior chest wall and then cannulated the pulmonary artery through an incision made in the free wall of the right ventricle. We cannulated the left atrium through an incision made at the left ventricular apex. We secured the cannulas in position with suture. We then perfused the pulmonary circulation with endothelial cell growth media supplemented with 4% (g/mL) bovine serum albumin at 1 mL/min flow. Perfusate was kept at 37°C with a water bath. We then added 0.5 U/mL of heparinase I and III mix to the perfusate and continued isogravimetric perfusion for 30 min. At the end of the experiment, we collected perfusate and stored it at -80°C until mass spectrometric analysis.

Measurement of glycocalyx thickness with intravital microscopy. We performed intravital microscopy as previously described (31).

Briefly, we anesthetized animals with ketamine and xylazine, and placed a glass window (coverslip) into the right anterior thoracic wall. We infused 150 kDa FITC-dextran into the jugular vein to serve as vascular tracer that does not penetrate the glycocalyx. We injected either heparinase I or III and captured pulmonary microvasculature for 1.5 h. We used a custom-designed intravital microscope (31) to simultaneously measure total vessel width (endothelial cell border to the opposite endothelial cell border, as defined by differential interference contrast microscopy) as well as FITC-dextran width (which does not include the glycocalyx). We determined glycocalyx thickness by subtracting the FITC-dextran width from the total vascular width and then dividing by two. At least three microvessels (<20- μ m width) were measured per each high-powered field.

FAC sorting of pulmonary endothelial cells. We excised the whole lung from each animal and finely minced lung tissue with a scalpel. We then digested the tissue with collagenase type 2 (1,000 U/mL) and dispase I (0.125 U/mL) as well as DNase I (0.01 Kunitz U/mL) in HBSS, with agitation for 60 min. We filtered digested tissue through a 70- μ m filter to remove undigested tissues and lysed erythrocytes with ACK lysing buffer for 3 min at 37°C. We washed cells with PBS supplemented with 4% fetal bovine serum and then stained with antibodies against CD31 and CD144 at 1:100 for 40 min at 4°C in dark. Live (determined by negative staining for DAPI), double positive population, i.e., CD31⁺/CD144⁺ endothelial cells, were then sorted and lysed immediately after the sorting in lysis buffer supplied in RNeasy Mini Kit. Total RNA was extracted immediately using RNeasy Mini Kit according to manufacturer's protocol. RNA integrity was tested with automated electrophoresis before further analysis.

RNA microarray. Total RNA from pulmonary endothelial cells (CD31⁺/CD144⁺) was used to synthesize cDNA, and cDNA was hybridized to a microarray (Mouse Clariom D; Affymetrix) and scanned with Affymetrix Genechip Scanner 3000. Samples with RNA integrity number >8.7 were used for analysis.

Quantitative reverse transcription-polymerase chain reaction. We synthesized cDNA using the iScript cDNA synthesis kit from total RNA isolated from FAC-sorted endothelial cells according to manufacturer's protocol. Using Taqman probes, we performed quantitative reverse transcription-polymerase chain reaction (qRT-PCR) with the Applied Biosystems 7300 Real-Time PCR System. Cyclophilin (whose expression was unchanged between sham and CLP groups, data not shown) was used as a housekeeping gene, and we analyzed data with the $2^{-\Delta\Delta C_t}$ method (17).

Production of recombinant HSulf-1 protein. Recombinant HSulf-1 was expressed as previously described (7). Briefly, HSulf-1 coding sequence was inserted in a pcDNA3.1/Myc-His(-) vector, between SNAP and 6His tags at the NH₂ and COOH terminus, respectively. This vector was used to stably transfect FreeStyle HEK 293-F cells. After harvesting the culture medium, we purified HSulf-1 using two steps of cation-exchange (SP-Sepharose) and size-exclusion chromatography (Superdex-200), as previously described for HSulf-2 (32). After purification, the protein was concentrated over a 30-kDa centrifugal unit, supplemented with 20% glycerol, aliquoted, and stored at -20°C.

Enzyme activity was assessed as previously described (33) by analyzing the disaccharide composition of untreated and Sulf-treated heparin in trisulfated [UA(2S)-GlcNS(6S)] disaccharide (substrate) and [UA-GlcNS(6S)] disulfated disaccharide (product), using reverse-phase ion-pair high-performance liquid chromatography (RIP-HPLC) coupled to 2-cyanoacetamide postcolumn fluorescent derivatization (8).

In vitro assays. We cultured primary human pulmonary microvascular endothelial cells using microvascular endothelial growth media. We knocked down Sulf-1 using siRNA targeted to Sulf-1, delivered with Lipofectamine RNAiMax. siRNA was transfected when cells were >80% confluent for 24 h. We harvested cell-extracted total RNA using RNeasy Mini kit according to the manufacturer's protocol. Cells that were passages 3–6 were used for the study.

Statistical analysis. Statistical analysis was performed with GraphPad Prism version 7.0. Single-comparison analyses were performed by *t* test; multiple comparisons were performed by one-way ANOVA with Tukey's post hoc multiple comparisons test. *P* < 0.05 was considered significant. The values are expressed as means \pm SD.

RESULTS

Postseptic animals demonstrate impaired inflammatory response to intratracheal LPS. To establish a model of postseptic impairment in pulmonary inflammatory responses, we performed cecal ligation and puncture (CLP) or sham surgery. Three days later, we administered 3 μ g/g intratracheal (IT) LPS to induce lung inflammation (Fig. 1A). Compared with sham-operated animals, post-CLP animals showed decreased alveolar leukocyte infiltration (Fig. 1B) and protein concentration (Fig. 1C) 2 days after intratracheal LPS. Lung histology from sham-operated animals demonstrated robust intratracheal LPS-induced lung inflammation, including cellular infiltration and alveolar flooding (Fig. 1D), while such pathology was absent in the lungs of post-CLP animals (Fig. 1E). Taken together, these findings are consistent with a murine model of CARS.

The postseptic, reconstituted pulmonary endothelial glycocalyx is remodeled. We have previously demonstrated that the pulmonary endothelial glycocalyx, degraded during early sepsis, is reconstituted by 72 h after CLP (45), a time point coincident with our observation of impaired pulmonary inflammatory responses (Fig. 1). As a simple screen for the presence of HS remodeling, we tested the sensitivity of the postseptic glycocalyx to heparinases with different preferences for sulfated domains of HS. Heparinase-I (which preferentially targets sulfated domains of HS; Ref. 3) readily degraded the postseptic and postsham glycocalyx (Fig. 2A). In contrast, heparinase-III, which targets undersulfated regions at the periphery of sulfated domains (4), was unable to degrade the postseptic glycocalyx (Fig. 2B). These findings suggested structural remodeling of the postseptic endothelial glycocalyx.

We then directly examined the disaccharide composition of HS comprising the reconstituted pulmonary endothelial glycocalyx (Fig. 2C). We perfused lungs isolated from mice 72 h after CLP (or sham) with both heparinase I and III for 30 min. We collected the perfusate and measured disaccharide sulfation patterns with HPLC-mass spectrometry multiple reaction monitoring, a high-sensitivity approach capable of detecting nanogram per milliliter concentrations of HS (30). HS extracted from the postseptic endothelial glycocalyx demonstrated increased 6-*O*-sulfation compared with HS isolated from the glycocalyx of sham mice (Fig. 2D). This postseptic increase in 6-*O*-sulfation was similarly observed to occur in HS fragments circulating in the blood of mice after CLP (Fig. 2E). Taken together, these findings suggested that sepsis alters the endothelial machinery responsible for HS disaccharide sulfation, favoring the presence of 6-*O*-sulfation within the pulmonary vasculature.

Pulmonary endothelial cells in postseptic animals have decreased expression of Sulf-1. To determine the presence of transcriptional changes in HS-modifying genes (potentially responsible for the observed increase in 6-*O*-sulfation detailed in Fig. 2), we performed FAC sorting to isolate pulmonary endothelial cells (CD31⁺/CD144⁺ population) from lungs harvested 48 h after CLP (i.e., immediately before remodeling) or

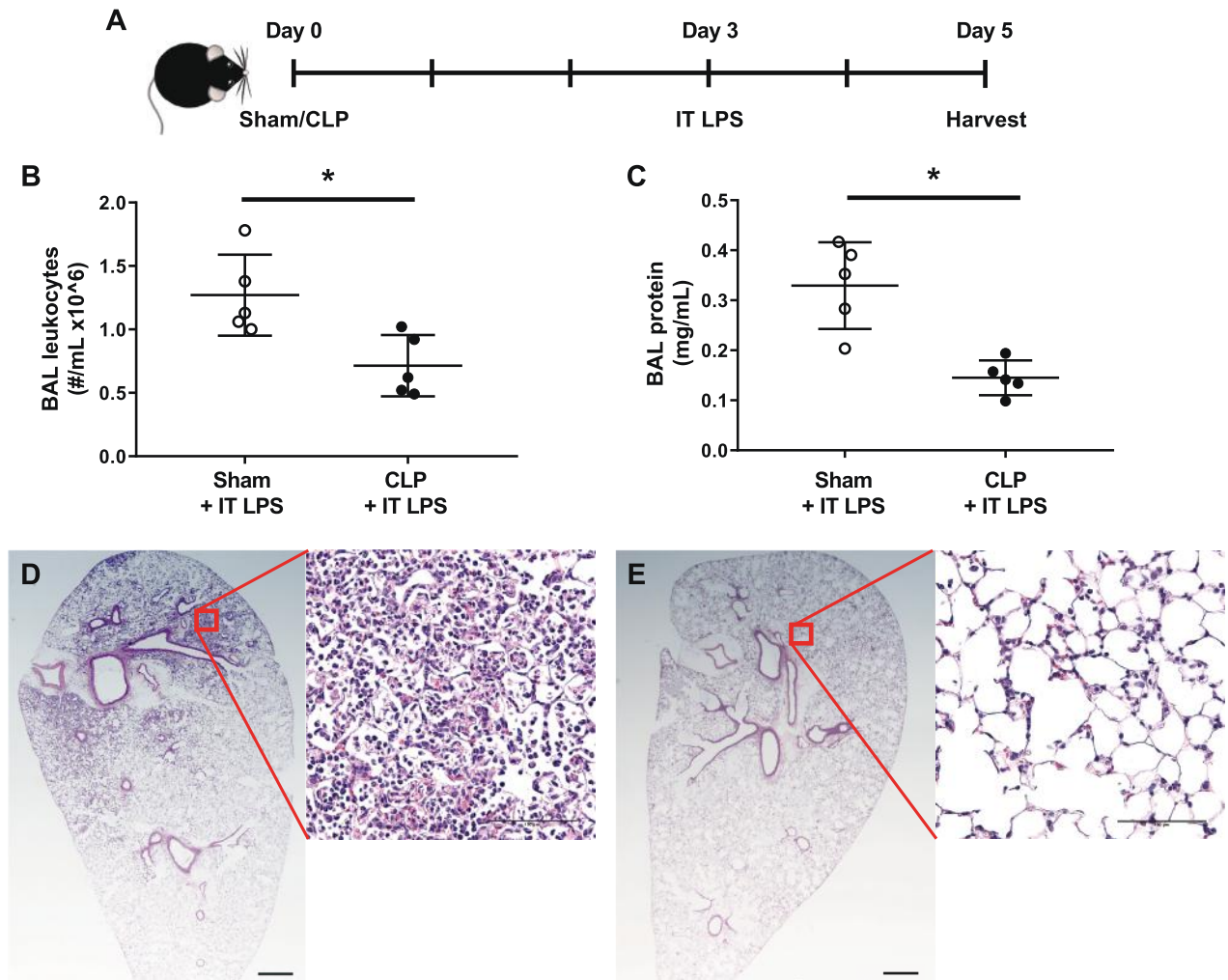


Fig. 1. Post-cecal ligation and puncture (CLP) mice demonstrate suppressed inflammatory response to intratracheal (IT) lipopolysaccharide (LPS). *A*: we performed CLP or sham surgery on *day 0*. Three days after surgery, we challenged animals with IT LPS ($3 \mu\text{g/g}$ body wt). Animals were harvested 2 days after IT LPS. *B*: post-CLP animals had decreased number of leukocytes per milliliter in bronchoalveolar lavage (BAL) fluid 2 days after IT LPS. *C*: post-CLP animals demonstrated less alveolar injury 2 days after IT LPS, based on protein concentrations in BAL fluid. *D*: lung histological section from a sham animal (stained with H&E) showed evidence of lung inflammation 2 days after IT LPS. *E*: lung histological section from a CLP animal (stained with H&E) 2 days after IT LPS showed minimal evidence of lung inflammation. Scale bars on lower-magnification images = $500 \mu\text{m}$ and those on higher-magnification images = $100 \mu\text{m}$. $*P < 0.05$ by Student's *t* test; $n = 5$ each group.

sham (Fig. 3A). Whole RNA transcriptome microarray analyses identified that postseptic pulmonary endothelial cells downregulated expression of Sulf-1 (Fig. 3B), an enzyme that constitutively removes 6-*O*-sulfo groups from extracellular HS-chains. Strikingly, there was no difference in other genes that modify 6-*O*-sulfation of HS, namely sulfatase-2 or HS 6-*O*-sulfotransferase (microarray data are publicly available with GEO accession number GSE129775). We confirmed these findings in a separate cohort of mice by repeating FAC sorting of pulmonary endothelial cells, then performing qRT-PCR of Sulf-1 (Fig. 3C).

To determine if downregulation of endothelial Sulf-1 similarly occurs after nonseptic causes of glycocalyx degradation, we enzymatically degraded endothelial HS from naïve mice by intravenous heparinase III injection (Fig. 3D). This model leads to rapid endothelial glycocalyx reconstitution within 24 h

(45). Twelve hours after heparinase-III (a time point before completion of reconstitution), qRT-PCR showed no difference in endothelial Sulf-1 mRNA expression level between heat-inactivated heparinase-III control and heparinase-III treatment groups (Fig. 3E). These findings suggest that downregulation of endothelial Sulf-1 is a sepsis-specific phenomenon.

Loss of Sulf-1 is necessary for postseptic suppression of pulmonary inflammatory responses to intratracheal LPS. Given that loss of endothelial Sulf-1 immediately preceded immunosuppression in postseptic animals, we determined whether loss of Sulf-1 was necessary for CARS. We produced recombinant, enzymatically active Sulf-1 as previously described (7). Resultant Sulf-1 was enzymatically active as evidenced by the 6-*O*-desulfation of trisulfated [UA(2S)-GlcNS(6S)] heparin disaccharides (NS2S6S) into [UA(2S)-GlcNS] disulfated disaccharides (NS2S), monitored by RPIP-

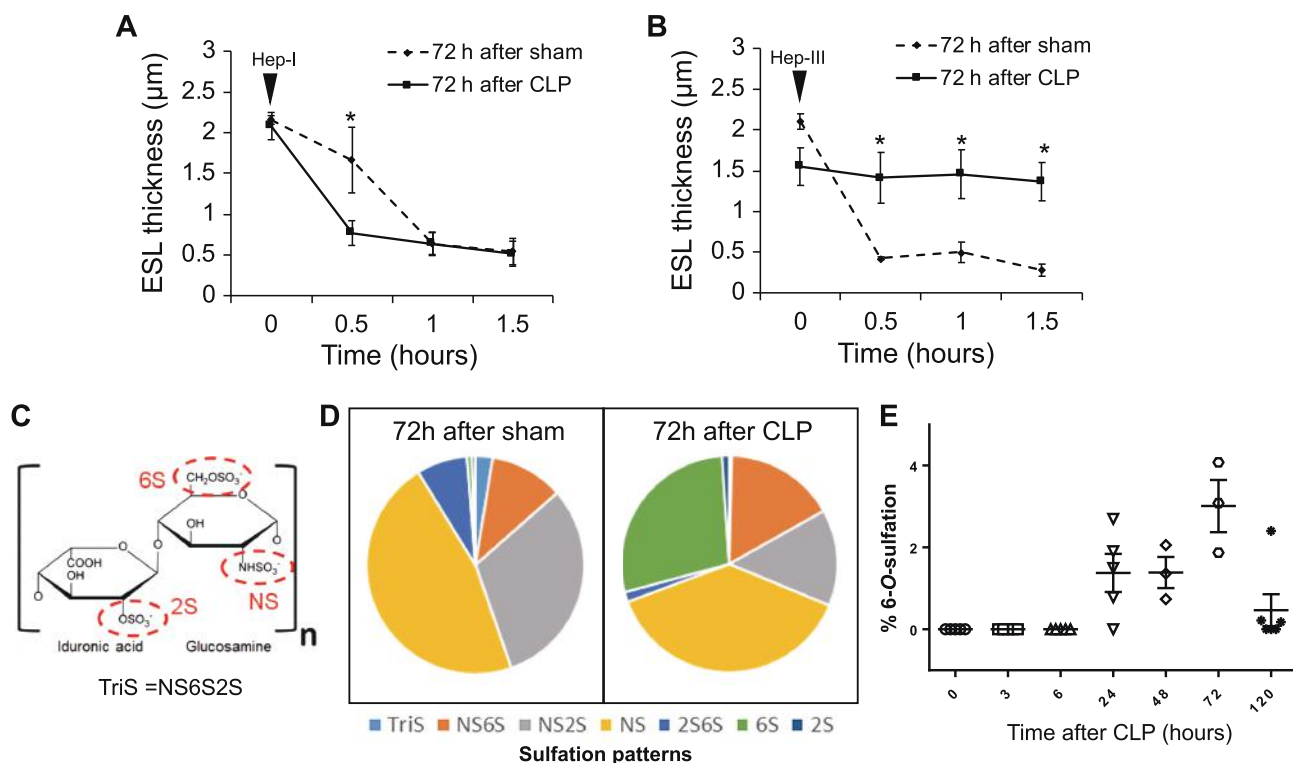


Fig. 2. The postseptic, reconstituted endothelial glycocalyx is remodeled. *A*: at the 72-h time point characterized by pulmonary endothelial heparan sulfate (HS) reconstitution, we injected 1 unit of heparinase I (Hep I) into the jugular vein, which degraded both sham and post-cecal ligation and puncture (CLP) pulmonary endothelial glycocalyx heparan sulfate. *B*: in contrast, 1 unit of heparinase III (Hep III) into the jugular vein did not degrade postseptic pulmonary endothelial heparan sulfate. *C*: major sulfation positions on heparan sulfate constituent disaccharides. *D*: postseptic, reconstituted pulmonary endothelial heparan sulfate had a significant increase ($P < 0.05$) in disaccharides with 6-*O*-sulfation (percentage of total sulfated disaccharides) as compared with heparan sulfate from sham animals. *E*: the percentage of 6-*O*-sulfated disaccharides increased over time in the plasma of post-CLP mice. ESL, endothelial surface layer. * $P < 0.05$ by Student's *t* test; $n = 3-5$ per group.

HPLC (Fig. 4A). We induced sepsis in mice by CLP and supplemented surviving animals with exogenous Sulf-1 intravenously (3- μ g bolus), 2 h before intratracheal (IT) LPS challenge (Fig. 4B). Compared with diluent control group, Sulf-1-treated animals had increased alveolar leukocyte (Fig. 4C) and protein (Fig. 4D) concentrations, suggesting reversal of postseptic immunosuppression. Lung histology showed a modest, scattered increase in leukocyte inflammation after IT LPS in post-CLP mice treated with intravenous Sulf-1 (Fig. 4, *E* and *F*). Of note, lungs from Sulf-1-treated animals showed marked perivascular cuffing (Fig. 4*F*, black arrows), suggesting restoration of an inflammatory edematous response to LPS. These results suggest that postseptic loss of Sulf-1 is necessary to suppress inflammatory responses to subsequent IT LPS.

Loss of Sulf-1 is not sufficient to induce immunosuppression in nonseptic animals. We created inducible, endothelial-specific Sulf-1 and Sulf-2 double knockout mice by breeding Sulf-1^{fl/fl} Sulf-2^{fl/fl} floxed mice with tamoxifen-inducible, endothelial-specific VECadCreERT2 mice to determine whether loss of endothelial Sulf-1 is sufficient to cause impaired pulmonary inflammation. Of note, as Sulf-2 is minimally expressed in the pulmonary endothelium (Ref. 24; confirmed by our whole transcriptome experiments, data not shown), endothelial-specific deletion of Sulf-2 is unlikely to impart significant pulmonary impact. We induced recombination with tamoxifen injections for 5 consecutive days (1 mg/day, ip). After

2 wk, we first confirmed inducible endothelial-specific recombination with FAC sorting followed by DNA electrophoresis (Fig. 5A). The cell-specific recombination was confirmed with DNA extracted from FAC-sorted endothelial cells (CD31⁺/CD144⁺) and nonendothelial cells (CD31⁻/CD144⁻) from Sulf-1^{fl/fl} Sulf-2^{fl/fl} VECadCreERT2⁺ or Sulf-1^{fl/fl} Sulf-2^{fl/fl} VECadCreERT2⁻ mice, both treated with tamoxifen (Fig. 5B). These knockout animals had normal blood leukocyte differential and normal coagulation, as measured by complete blood counts and thromboelastography clot onset time (R-time), respectively (data not shown). There was no evidence of increased pulmonary apoptosis in these mice after tamoxifen treatment (TUNEL staining), and lung histology was unchanged (data not shown). We performed intravital microscopy to determine if the sensitivity of the pulmonary endothelial glycocalyx of tamoxifen-treated, Sulf-1^{fl/fl} Sulf-2^{fl/fl} VECadCreERT2⁺ mice to enzymatic degradation was similar to that observed in postseptic wild-type mice (Fig. 2*B*). The pulmonary endothelial glycocalyx of these Sulf-1 and -2 double knockout mice demonstrated resistance to heparinase III (Fig. 5C) but not against heparinase I (data not shown), confirming similar glycocalyx remodeling as observed in postseptic mice (Fig. 2. *A* and *B*).

We challenged tamoxifen-treated Sulf-1^{fl/fl} Sulf-2^{fl/fl} VECadCreERT2⁺ mice with intratracheal LPS to determine whether endothelial-specific Sulf-1/Sulf-2 knockout is sufficient to

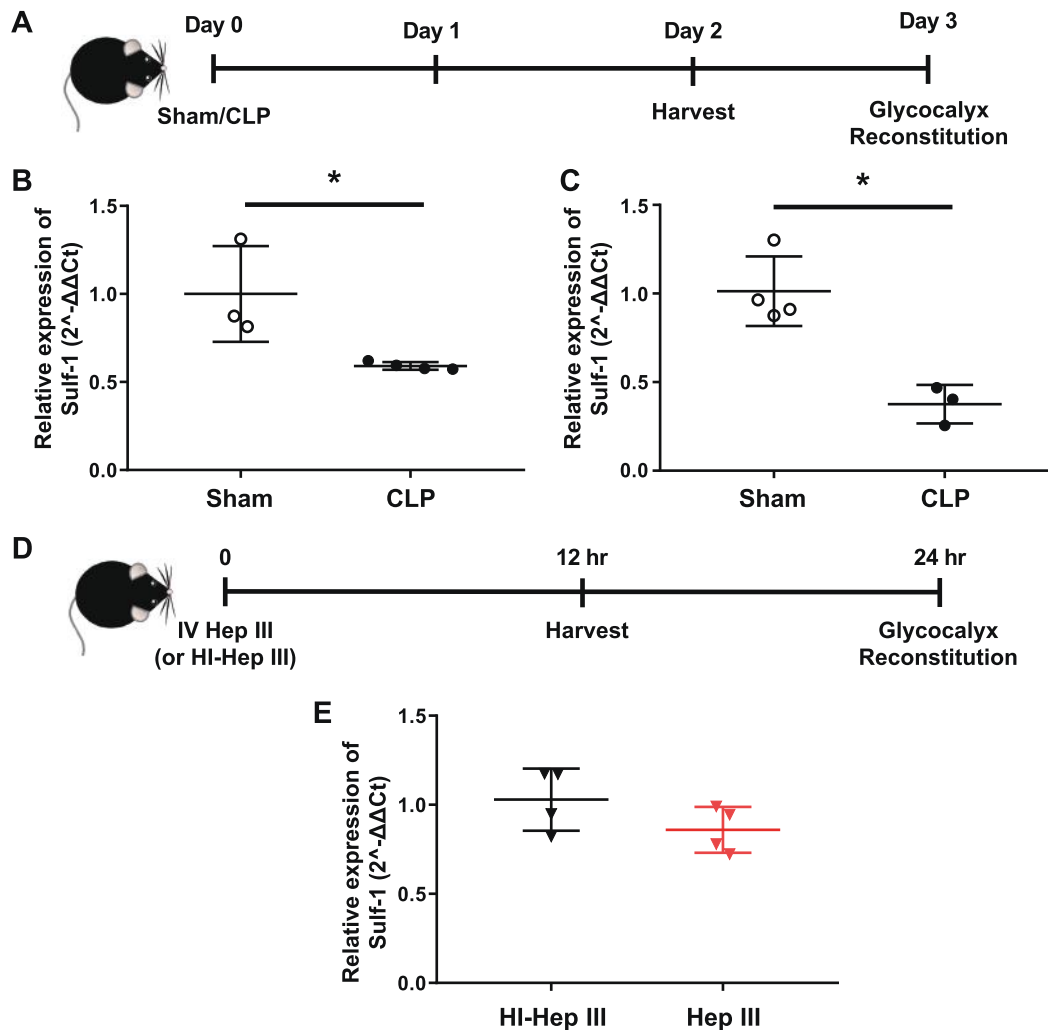


Fig. 3. Postseptic pulmonary endothelial cells downregulate sulfatase-1 (Sulf-1) mRNA. *A*: we collected pulmonary endothelial cells and extracted total RNA at *day 2*, a time point immediately before glycocalyx reconstitution at *day 3*. *B*: pulmonary endothelial cells in mice 48 h after cecal ligation and puncture (CLP) had decreased Sulf-1 expression detected with RNA microarray. *C*: we validated the downregulation of Sulf-1 in pulmonary endothelial cells by quantitative RT-PCR using separate biological replicates. *D*: we collected pulmonary endothelial cells and extracted total RNA at 12 h after heparinase III (Hep III) injection, a time point immediately before glycocalyx reconstitution after enzymatic, nonseptic degradation. IV, intravenous. *E*: mRNA expression level of Sulf-1 in pulmonary endothelial cells were similar 12 h after enzymatic degradation by Hep III or heat-inactivated (HI) Hep III. * $P < 0.05$ by Student's *t* test; $n = 3-4$ each group.

impair pulmonary inflammatory responses in nonseptic mice, (Fig. 5A). There was no difference between control group animals and double knockout animals in leukocytes numbers (Fig. 5D) and protein concentration (Fig. 5E) in BAL fluid. Similarly, there was no obvious difference observed in lung histology between the control group (Figs. 5F) and Sulf-1/2 knockout group (Fig. 5G). Taken together, the data indicated that loss of Sulf-1 is not sufficient to cause impaired pulmonary inflammation in non-septic animals.

Sulf-1 silencing in human primary pulmonary microvascular endothelial cells results in decreased mRNA expression of ICAM-1. 6-*O*-sulfation may impact numerous biological pathways, spanning growth factor, chemokine, and damage-associated molecular pattern signaling (5). We used siRNA approaches to knockdown Sulf-1 in primary human microvascular endothelial cells to determine if the consequences of Sulf-1

loss on these signaling pathways converge to impart a general anti-inflammatory phenotype to endothelial cells. We cultured primary human lung microvascular endothelial cells in a six-well plate and transfected siRNA using lipofectamine. We performed three biological replicates, each of which represented cells collected from a separate human donor. Our transfection resulted in >83% knockdown efficiency (Fig. 6A). We examined whether a major adhesion molecule for neutrophil adhesion, ICAM-1, was affected by this knockdown. Loss of Sulf-1 resulted in downregulation of ICAM-1 expression in endothelial cells (Fig. 6B). These findings suggest that loss of Sulf-1 may drive pathways that suppress endothelial activation. As these unstimulated endothelial cells expressed little ICAM-1 protein at baseline (data not shown), we are unable to demonstrate a direct effect of loss of Sulf-1 on endothelial-leukocyte adhesion.

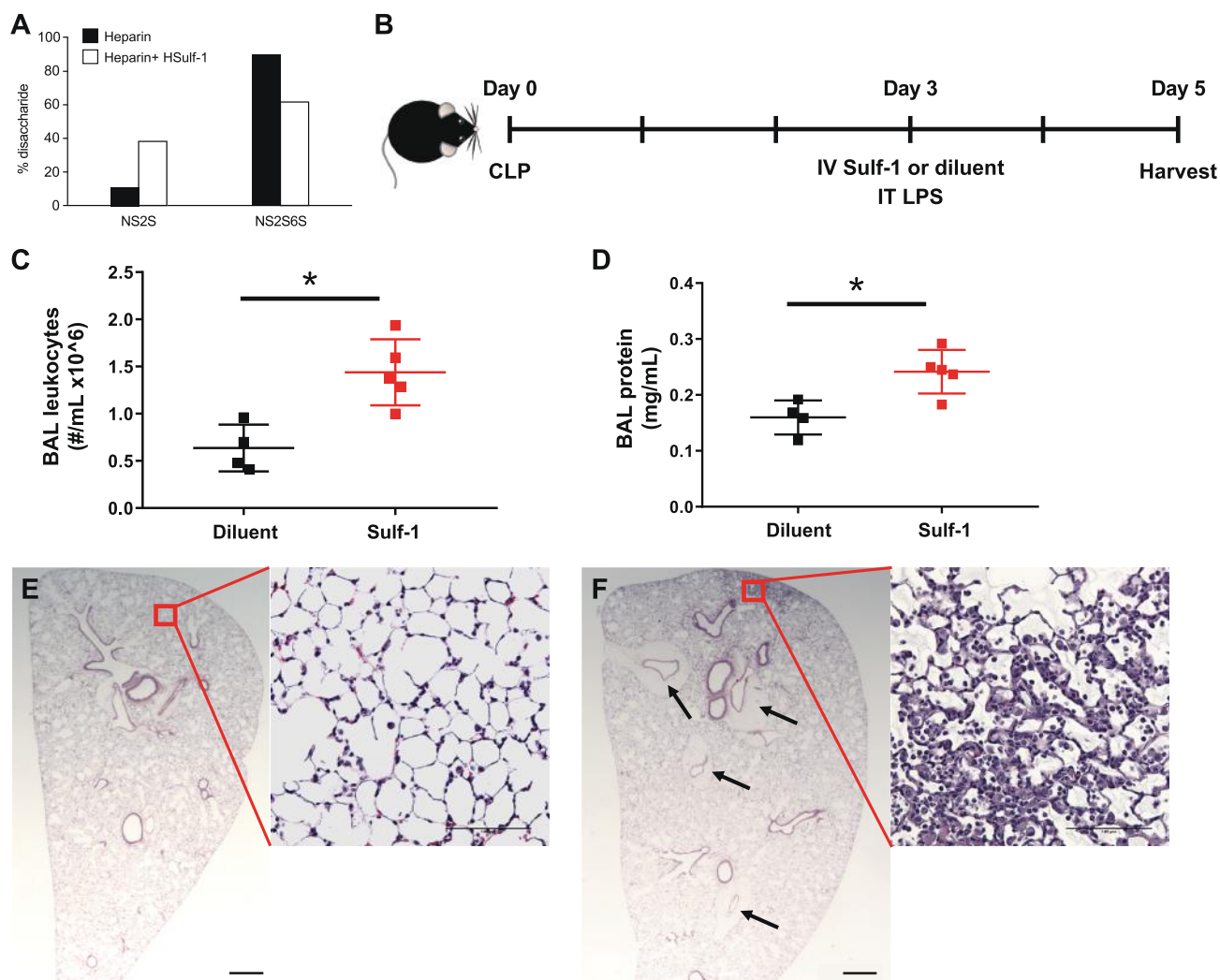


Fig. 4. Loss of sulfatase-1 (Sulf-1) contributes to postseptic compensatory anti-inflammatory response syndrome. *A*: recombinant Sulf-1 was enzymatically active and efficiently removed 6-*O* sulfates from heparin, as shown by the decrease in trisulfated [UA(2S)-GlcNS(6S)] disaccharide content (NS2S6S) and concomitant increase of [UA-GlcNS(6S)] disulfated disaccharide (NS2S) in Sulf-1-treated heparin (white bars) compared with untreated control (black bars). *B*: we performed cecal ligation and puncture (CLP) on mice on *day 0*. On *day 3*, surviving postseptic mice were treated with intravenous (IV) Sulf-1 (3 μ g bolus) or diluent, then challenged with intratracheal (IT) LPS (3 μ g/g body wt). Animals were harvested 2 days after IT LPS. *C*: Sulf-1-treated animals had increased leukocytes in bronchoalveolar lavage (BAL) fluid 2 days after IT LPS, as compared with diluent treated animals 2 days after IT LPS. *D*: Sulf-1-treated animals also showed increased lung injury based on protein concentration in BAL fluid. *E*: lung histological section from a postseptic, diluent-treated animal (stained with H&E) showed very little evidence of lung inflammation. *F*: lung histological section from a postseptic Sulf-1-treated animal (stained with H&E) showed increased tissue consolidation 2 days after IT LPS. In addition, Sulf-1-treated animals had marked perivascular cuffs (black arrows). Scale bars on lower-magnification images = 500 μ m and those on higher-magnification images = 100 μ m. **P* < 0.05 by Student's *t* test; *n* = 4–5 each group.

DISCUSSION

In this study, we demonstrated that post-CLP mice have impaired alveolar inflammation in response to intratracheal LPS. This postseptic “immunoparalysis” coincides with reconstitution of a remodeled pulmonary endothelial glycocalyx, as demonstrated by differential sensitivity to enzymatic degradation during intravital microscopy and enrichment in 6-*O*-sulfated HS monitored by mass spectrometry. These changes coincide with selective pulmonary endothelial downregulation of Sulf-1, an enzyme dedicated to the constitutive removal of 6-*O*-sulfo groups. We observed that loss of Sulf-1 is necessary for impaired inflammation in postseptic animals, but it alone

was not sufficient to cause impaired inflammation in nonseptic animals. Our data therefore collectively indicate an endothelial role in CARS, a syndrome to date largely relegated to post-septic leukocyte dysfunction.

Our findings indicate that sepsis suppresses endothelial Sulf-1, impairing subsequent inflammatory responses after the resolution of septic endothelial injury. Teleologically, this response may be designed to limit the degree of lung injury, shifting endothelial signaling toward tissue repair. For example, an increase in 6-*O*-sulfation promotes cell response to HS-binding growth factors, FGF2 and VEGF, which may promote endothelial repair processes (6). However, this re-

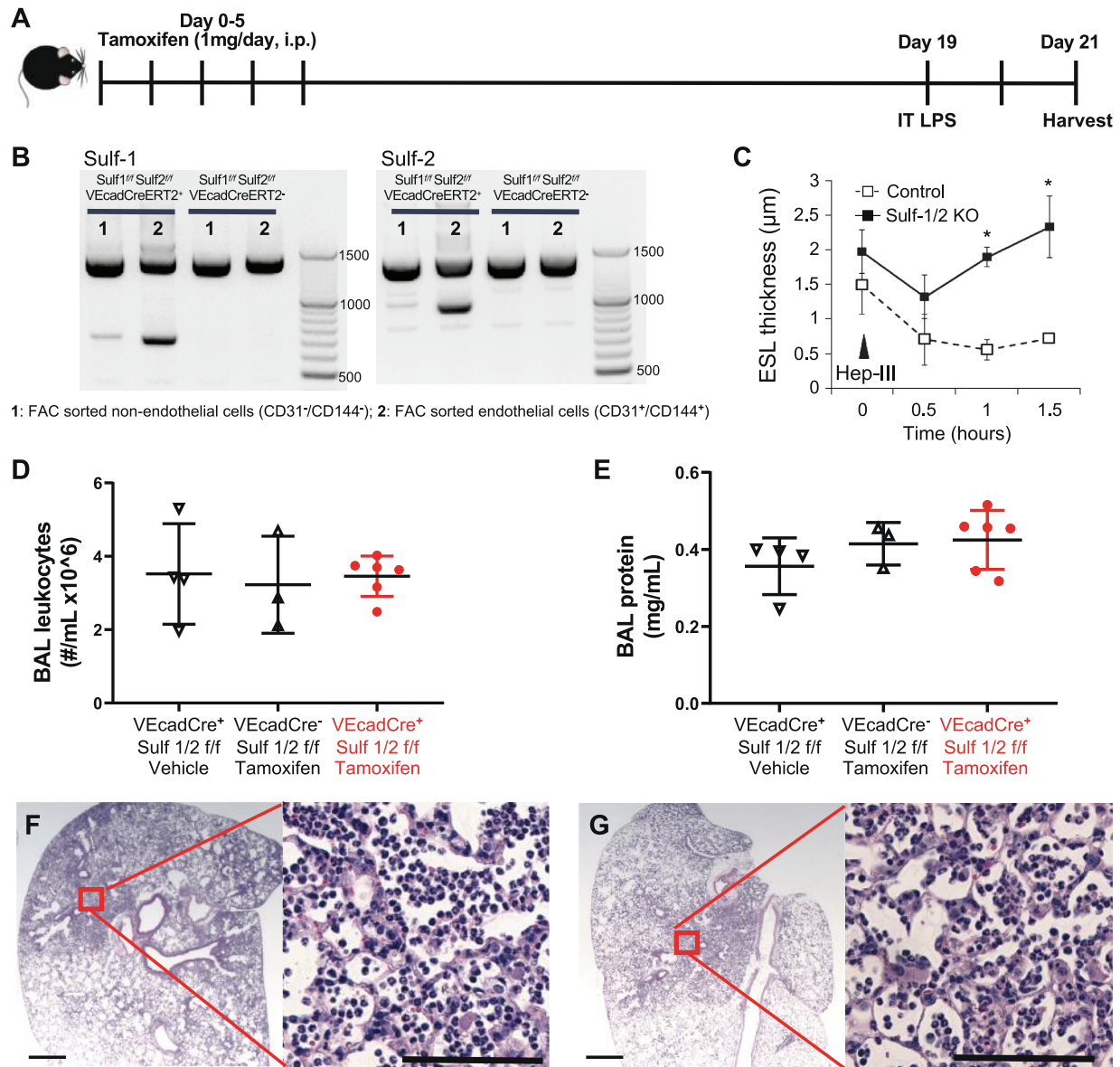


Fig. 5. Loss of sulfatase-1 (Sulf-1) is not sufficient to cause impaired inflammation in nonseptic animals. **A:** Sulf-1^{fl/fl} Sulf-2^{fl/fl} VECadCreERT2⁺ or ⁻ animals received tamoxifen or vehicle control injections intraperitoneally for 5 consecutive days (1 mg/day). Recombination of genes and pulmonary endothelial glycoalkal characteristics were evaluated 2 wk after the last injection of tamoxifen (or vehicle). Knockout or control mice were alternatively challenged with intratracheal (IT) LPS (3 μ g/g body wt) at the same time point. **B:** we confirmed cell-specific, inducible recombination of Sulf-1 and Sulf-2 with DNA gels. *Lane 1:* DNA from pulmonary nonendothelial cells (CD31⁻/CD144⁻). *Lane 2:* DNA from pulmonary endothelial cells (CD31⁺/CD144⁺). **C:** pulmonary endothelial glycoalkal of Sulf-1/2 knockout animals was resistant to heparinase III (Hep III) degradation, similar to the postseptic endothelial glycoalkal resistance to heparinase III observed in wild-type mice (Fig. 2B). Control animals used were Sulf-1^{fl/fl} Sulf-2^{fl/fl} VECadCreERT2⁻ (floxed gene alone without Cre recombinase), treated with tamoxifen. **D:** number of leukocytes in bronchoalveolar lavage (BAL) fluid in Sulf-1/2 knockout animals did not differ from control groups 2 days after IT LPS. **E:** protein concentration of BAL fluid was similarly not different among the experimental groups. **F:** lung histological section from a control animal (Sulf-1^{fl/fl} Sulf-2^{fl/fl} VECadCreERT2⁻, treated with tamoxifen) had clear consolidation. **G:** lung histological section from a Sulf-1/2 knockout animal similarly had evidence of lung inflammation. Scale bars on lower-magnification images = 500 μ m and those on higher-magnification images = 100 μ m. **P* < 0.05 by one-way ANOVA with post hoc Tukey test; *n* = 3–6 each group.

sponse may backfire if the host is exposed to a secondary infection by attenuating host-protective antimicrobial responses. Postseptic patients are susceptible to secondary infection, with hospital-acquired pneumonia the most common complication (15, 39). Septic patients who later acquired secondary infections experience more severe illness, longer

length-of-stay, and higher 1-yr mortality (39, 40). Therefore, in the presence of hospital-acquired infection, loss of endothelial Sulf-1 may be detrimental. Additionally, we found that postseptic animals had increased absolute neutrophil count in blood at the time of intratracheal LPS (data not shown), indicating that decreased neutrophil infiltration found in BAL fluid is not

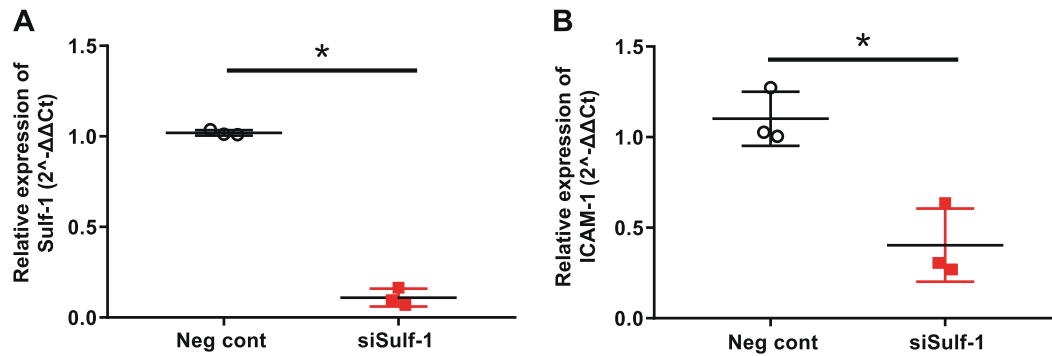


Fig. 6. Knocking down sulfatase-1 (Sulf-1) in pulmonary microvascular endothelial cells decreases ICAM-1 expression. *A*: we transfected cultured endothelial cells with siRNA targeted to Sulf-1 using Lipofectamine RNAiMAX for 24 h, which resulted in greater than 83% knockdown of Sulf-1. *B*: knockdown of Sulf-1 resulted in downregulation of ICAM-1 expression in endothelial cells. Control cells were treated with lipofectamine without siRNA. **P* < 0.05, Student's *t* test; *n* = 3, each data point represents average of 2–3 wells per treatment.

due to postseptic depletion of circulating neutrophils. Interestingly, when the whole transcriptome profiles from circulating leukocytes were compared between patients who acquired secondary infection and those who did not, there was no difference in pro- or anti-inflammatory genes (39). However, there was significant increase in plasma proteins, including circulating cytokines, such as IL-8 and IL-10, and markers of vascular dysfunction and activation, such as E-selectin, angiopoietins, ICAM-1, and proteins that promote coagulation in patients who developed secondary infections, compared with the patients who did not (40). Taken together, these findings suggest that CARS is most likely more complex than leukocyte dysfunction and warrant further investigations to determine contributions by other cells and organs such as vascular endothelial cells.

Although we have shown one potential effect of loss of Sulf-1, i.e., endothelial ICAM-1 downregulation, the mechanisms by which loss of Sulf-1 results in impaired inflammation remain uncertain. Sulf-1 and Sulf-2 are unique in that they are the only extracellular sulfatases modifying HS at the postsynthetic level, suggesting a critical importance of 6-*O*-sulfation for the function of glycocalyx HS. Indeed, 6-*O*-sulfation may influence numerous biological processes of consequence, including growth factor, chemokine, and damage-associated molecular pattern signaling (5). One possible mechanism is that a shift in 6-*O*-sulfation affects the affinity of endothelial HS to cytokines that modulate neutrophil infiltration. Sulf-2 is shown to selectively mobilize not only specific growth factors but also cytokines (such as SDF-1 and SLC) that impact neutrophil migration (36, 38). Changes in endothelial HS 6-*O*-sulfation shape the microenvironment and inflammatory response in multiple ways, given their multiple biological roles. Changes in HS sulfation may result in altered systemic immunity and enhanced bacterial adhesion to endothelial cells (43). Although the roles of endothelial selectins to neutrophil extravasation in the pulmonary circulation are complex (13, 20), it has been reported that changes in sulfation in endothelial HS, particularly 6-*O*-sulfation, weaken neutrophil binding to L-selectin and P-selectin (41, 42). Accordingly, the immunosuppressive effects of loss of Sulf-1 may arise from numerous potential processes, potentially converging upon pathways such as adhesion molecule expression (Fig. 6). Future studies will screen for 6-*O*-sulfated HS-binding proteins, providing greater insight

into the downstream mechanisms responsible for our observed effects of Sulf-1 on postseptic pulmonary inflammation.

An additional consideration is that changes in endothelial Sulf-1/2 expression may impact the dynamics of glycocalyx degradation. Indeed, our endothelial microarray experiments (GEO accession no. GSE129775) demonstrated that postseptic (48 h) loss of Sulf-1 coincided with increased expression of matrix metalloproteinases 8, 9, and 25 and disintegrin and metalloproteinase domain-containing proteins 8, 15, and 23. No changes were seen in endothelial expression of heparanase or matrix metalloproteinase 15 in these postseptic mice. Future studies will be required to investigate the presence of sulfatase-sheddase cross talk and its relevance to lung injury and repair.

Sulf-1 and Sulf-2 preferentially targets trisulfated NS2S6S disaccharides of HS and, to a lesser extent, NS6S (22). Loss of Sulf-1, therefore, would be expected to result in an increase of trisulfated NS2S6S disaccharides (substrate), with a decrease in NS2S (product). However, we rather observed an increase of HS overall 6-*O*-sulfation (Fig. 2*D*). This discrepancy may reflect the additional ability of Sulf-1 and Sulf-2 to directly regulate HS biosynthesis enzymes (14). Therefore, it is possible that our biological observations following Sulf-1 downregulation may be caused directly by the loss of Sulf-1 activity, and/or indirectly, through a regulation of HS biosynthesis. Of note, our recombinant Sulf-1 is of human origin. Based on the strong sequence homology of HSulf-1 and MSulf-1, we do not expect any significant differences in enzymatic activity (22), but we cannot exclude the possibility that enzymatic activity is slightly different *in vivo*.

In conclusion, our study showed that postseptic pulmonary endothelial glycocalyx HS undergoes structural remodeling, coincident with loss of endothelial Sulf-1. Although loss of Sulf-1 in endothelial cells is not sufficient to cause impaired inflammation in non-septic mice, it contributes to postseptic CARS, offering a potential new target for treating postseptic patients at risk for nosocomial pneumonia.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

K.O., R.R.V., R.J.L., and E.P.S. conceived and designed research; K.O., X.H., Y.O., R.e.M., Y.Y., S.M.H., S.A.M., T.C.L., P.D.-C., F.Z., X.Y., R.R.V., R.J.L., and E.P.S. performed experiments; K.O., X.H., Y.O., R.e.M., Y.Y., S.M.H., S.A.M., T.C.L., P.D.-C., F.Z., X.Y., R.R.V., R.J.L., and E.P.S. analyzed data; K.O., X.H., S.A.M., X.Y., R.R.V., R.J.L., and E.P.S. interpreted results of experiments; K.O., R.J.L., and E.P.S. prepared figures; K.O., R.J.L., and E.P.S. drafted manuscript; K.O., Y.Y., F.Z., X.Y., R.R.V., R.J.L., and E.P.S. edited and revised manuscript; K.O., X.H., Y.O., R.e.M., Y.Y., S.M.H., S.A.M., T.C.L., P.D.-C., F.Z., X.Y., R.R.V., R.J.L., and E.P.S. approved final version of manuscript.

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