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

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

Abbreviated title: Endothelial loss of Sulf-1 and post-septic CARS

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35

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Abstract

Sepsis patients are at increased risk for hospital-acquired pulmonary infections, potentially due to post-septic 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. 72 hours after experimental sepsis, coincident with glycocalyx reconstitution, mice demonstrated impaired neutrophil and protein influx in response to intratracheal lipopolysaccharide (LPS). The post-septic 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 post-septic mice restored the pulmonary response to LPS, suggesting that loss of Sulf-1 was necessary for post-septic 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

61 Sulf-1 is necessary for post-septic suppression of pulmonary inflammation, representing
62 a novel endothelial contributor to CARS.

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 hyper-inflammation. However, the failures of numerous clinical trials targeting inflammatory signaling (18) led to the proposed concept that sepsis is not simply hyper-inflammation, 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 post-septic CARS includes known associations between mortality and increased plasma IL-10 and decreased HLA-DR expression on leukocytes (10, 16, 21), decreased production of cytokines, such as TNFα, IFN-γ, IL-6, IL-10 in septic patients, and depletion of CD4+, CD8+, HLA-DR+ cells in the spleen (2). CARS has therefore been largely attributed to dysfunctional monocytes, including impaired cytokine production, 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 post-septic impairment in lung inflammation.

The endothelial glycocalyx is 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 (glucuronic 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 hours after cecal ligation and puncture (CLP) in mice), endothelial synthesis of new HS allows for glycocalyx reconstitution, mediating endothelial recovery (45). We postulated that post-septic 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 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 non-septic animals. Knock down of Sulf-1 using siRNA in pulmonary microvascular endothelial cells resulted

in downregulation of ICAM-1 transcription. Our study therefore identifies post-septic remodeling of the pulmonary endothelial glycocalyx as a novel contributor to CARS.

Materials and Method

Materials

We purchased LPS (from *Escherichia coli* O55:B5), heparinase I and III (from *Flavobacterium heparinum*) from Sigma-Aldrich (St. Louis, MO) and reconstituted in phosphate buffered saline (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 in corn oil with 5% ethanol. For tissue digestion and fluorescence activated cell sorting, we purchased collagenase type 2 from Worthington Biochemical Corporation (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), and 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 transfection reagent, Lipofectamine RNAiMAX, from Thermo Fisher Scientific (Waltham, MA). For production of recombinant 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 of National Institutes of Health guideline. Wild type, male C57BL/6J mice (8-10 weeks) were purchased from Jackson Laboratory. Sulf-1 and 2 double floxed mice (both males and females, 8-12 weeks old) were used as previously described (37). VE-cadherin CRE-ERT2 mice were provided by Dr. Ralf Adams at Max Planck Institute, Germany. Sulf1/2 double homozygous mice with or without VE-cadherin-CRE-ERT2 (Sulf-$1^{f/f}$ Sulf-$2^{f/f}$ VEcadCreERT2$^{-\ or+}$) were used for all knock out 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 8-14 week-old mice with CLP as previously described (31). Briefly, we anesthetized animals with 5% isoflurane inhalation. An incision of approximately 1 cm was made in abdomen and cecum was exposed. We ligated the cecum at 50 % of its length and punctured it through and through with a 22 G needle. The cecum was then returned to the abdominal cavity, and the incision was closed with suture. Buprenorphine (1.6 µg/ g body weight, i.p.) 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 (CBCs) 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 (Haemonetics, 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 1000 x *g* for 10 min. and stored it at -80 °C until analysis. We cannulated the trachea and performed bronchoalveolar lavage (BAL) 3 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 N_2 and stored at -80 ºC 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 mL in BAL fluid using a hemocytometer. We centrifuged BAL fluid at 1200 rpm for 5 min. and stored the supernatant at -80 ºC until analysis. We determined total protein concentration in BAL fluid with a Coomassie Brilliant Blue-based colorimetric assay.

178 Cells in BAL fluid were mounted on a slide with cytospin at 600 rpm for 2 min., and
179 stained with Wright-Giemsa method for differential cell counting.

180 **Isolated perfused mouse lung**

181 We performed the isolated, perfused mouse lung as previously described (29). Briefly,
182 we deeply anesthetized mice with ketamine and xylazine. After confirming the loss of
183 toe-pinch reflex, we cannulated the trachea and ventilated mice with 21% O_2 and 5%
184 CO_2 at 125 breaths/min at tidal volume of 250 μL. We rapidly removed the sternum and
185 anterior chest wall, and then cannulated the pulmonary artery through an incision made
186 in the free wall of the right ventricle. We cannulated the left atrium through an incision
187 made at the left ventricular apex. We secured the cannulas in position with suture. We
188 then perfused the pulmonary circulation with endothelial cell growth media
189 supplemented with 4% (g/mL) bovine serum albumin at 1 mL/min flow. Perfusate was
190 kept at 37ºC with a water bath. We then added 0.5 unit/mL of heparinase I and III mix
191 to the perfusate and continued isogravimetric perfusion for 30 min. At the end of the
192 experiment, we collected perfusate and stored it at -80ºC until mass spectrometric
193 analysis.

194 **Measurement of glycocalyx thickness with intravital microscopy**

195 We performed intravital microscopy as previously described (31). Briefly, we
196 anesthetized animals with ketamine and xylazine, and placed a glass window
197 (coverslip) into the right anterior thoracic wall. We infused 150 kDa FITC-dextran into
198 the jugular vein to serve as vascular tracer that does not penetrate the glycocalyx. We
199 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, then dividing by two. At least three microvessels (< 20 μm width) were measured per each high-powered field.

Fluorescent Activated Cell (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 (1000 units/mL) and dispase I (0.125 units/mL) as well as DNase I (0.01 Kunitz units/mL) in HBSS, with agitation for 60 min. We filtered digested tissue through 70 μm filter to remove undigested tissues, and lysed erythrocytes with ACK lysing buffer for 3 min. at 37 ºC. We washed cells with PBS supplemented with 4% fetal bovine serum, then stained with antibodies against CD31 and CD144 at 1:100 for 40 min. at 4ºC 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 ($CD31^+/CD144^+$) were 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 greater than 8.7 were used for analysis.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

We synthetized 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 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 $2^{\wedge(-\Delta\Delta Ct)}$ 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 N- and C-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 RPIP-HPLC coupled to 2-cyanoacetamide post-column 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 cells extracted total RNA using RNeasy Mini kit according to manufacturer's protocol. Cells that were passage 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*-values less than 0.05 were considered significant. The values are expressed as mean ± SD.

Results

Post-septic animals demonstrate impaired inflammatory response to intratracheal LPS

To establish a model of post-septic impairment in pulmonary inflammatory responses, we performed cecal ligation and puncture (CLP) or sham surgery. Three days later, we administered 3 μg/g intratracheal (IT) LPS to induce lung inflammation (**Figure 1A**). Compared to sham-operated animals, post-CLP animals showed decreased alveolar leukocyte infiltration (**Figure 1B**) and protein concentration (**Figure 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 (**Figure 1D**), while such pathology was absent in the lungs of post-CLP animals (**Figure 1E**). Taken together, these findings are consistent with a murine model of CARS.

The post-septic, 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 (**Figure 1**). As a simple screen for the presence of HS remodeling, we tested the sensitivity of the post-septic glycocalyx to heparinases with different preferences for sulfated domains of HS. Heparinase-I (which preferentially targets sulfated domains of HS (3)) readily degraded the post-septic and post-sham glycocalyx (**Figure 2A**). In contrast, heparinase-III, which targets undersulfated regions at the periphery of sulfated domains (4), was

unable to degrade the post-septic glycocalyx (**Figure 2B**). These findings suggested structural remodeling of the post-septic endothelial glycocalyx.

We then directly examined the disaccharide composition of HS comprising the reconstituted pulmonary endothelial glycocalyx (**Figure 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 ng/ml concentrations of HS (30). HS extracted from the post-septic endothelial glycocalyx demonstrated increased 6-*O*-sulfation compared to HS isolated from the glycocalyx of sham mice (**Figure 2D**). This post-septic increase in 6-*O*-sulfation was similarly observed to occur in HS fragments circulating in the blood of mice after CLP (**Figure 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 post-septic 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 **Figure 2**), we performed FAC sorting to isolate pulmonary endothelial cells ($CD31^+/CD144^+$ population) from lungs harvested 48 h after CLP (i.e. immediately prior to remodeling) or sham (**Figure 3A**). Whole RNA transcriptome microarray analyses identified that post-septic pulmonary endothelial cells downregulated expression of sulfatase-1 (Sulf-1) (**Figure 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 publically 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 (**Figure 3C**).

To determine if downregulation of endothelial Sulf-1 similarly occurs after non-septic causes of glycocalyx degradation, we enzymatically degraded endothelial HS from naïve mice by intravenous heparinase III injection (**Figure 3D**). This model leads to rapid endothelial glycocalyx reconstitution within 24 h (45). Twelve hours after heparinase-III (a time point prior to 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 (**Figure 3E**). These findings suggest that downregulation of endothelial Sulf-1 is a sepsis-specific phenomenon.

Loss of Sulf-1 is necessary for post-septic suppression of pulmonary inflammatory responses to intratracheal LPS

Given that loss of endothelial Sulf-1 immediately preceded immunosuppression in post-septic 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**)-Glc**NS**(**6S**)] heparin disaccharides (NS2S6S) into [UA(**2S**)-Glc**NS**] disulfated disaccharides (NS2S), monitored by RPIP-HPLC (**Figure 4A**). We induced sepsis in mice by CLP and supplemented surviving animals with exogenous Sulf-1 intravenously (3 μg bolus), 2 h prior to intratracheal (IT) LPS challenge (**Figure 4B**).

Compared to diluent control group, Sulf-1 treated animals had increased alveolar leukocyte (**Figure 4C**) and protein (**Figure 4D**) concentrations, suggesting reversal of post-septic immunosuppression. Lung histology showed a modest, scattered increase in leukocyte inflammation after IT LPS in post-CLP mice treated with intravenous Sulf-1 (**Figure 4E**, **4F**). Of note, lungs from Sulf-1 treated animals showed marked perivascular cuffing (**Figure 4F**, black arrows), suggesting restoration of an inflammatory edematous response to LPS. These results suggest that post-septic 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 non-septic animals

We created inducible, endothelial-specific, Sulf-1 and Sulf-2 double knockout mice by breeding Sulf-$1^{f/f}$ Sulf-$2^{f/f}$ 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 ((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, i.p.). After 2 weeks, we first confirmed inducible endothelial-specific recombination with FAC sorting followed by DNA electrophoresis (**Figure 5A**). The cell-specific recombination was confirmed with DNA extracted from FAC sorted endothelial cells ($CD31^+$/$CD\ 144^+$) and non-endothelial cells ($CD31^-$/$CD\ 144^-$) from Sulf-$1^{f/f}$ Sulf-$2^{f/f}$ $VEcadCreERT2^+$ or Sulf-$1^{f/f}$ Sulf-$2^{f/f}$ $VEcadCreERT2^-$ mice, both treated with tamoxifen (**Figure 5B**). These knockout animals had normal blood

leukocyte differential and normal coagulation, as measured by CBCs 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$^{f/f}$ Sulf-2$^{f/f}$ VEcadCreERT2^{+} mice to enzymatic degradation was similar to that observed in post-septic wild-type mice (**Figure 2B**). The pulmonary endothelial glycocalyx of these Sulf-1 and -2 double knockout mice demonstrated resistance to heparinase-III (**Figure 5C**), but not against heparinase I (data not shown), confirming similar glycocalyx remodeling as observed in post-septic mice (**Figure 2A, 2B**).

We challenged tamoxifen-treated Sulf-1$^{f/f}$ Sulf-2$^{f/f}$ VEcadCreERT2^{+} mice with IT LPS to determine whether endothelial-specific Sulf-1/Sulf-2 knockout is sufficient to impair pulmonary inflammatory responses in non-septic mice, (**Figure 5A**). There was no difference between control group animals and double knockout animals in leukocytes numbers (**Figure 5D**) and protein concentration (**Figure 5E**) in BAL fluid. Similarly, there was no obvious difference observed in lung histology between the control group (**Figures 5F**) and Sulf-1/2 knockout group (**Figure 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 knock down 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 6-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 greater than 83% knock down efficiency (**Figure 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 (**Figure 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.

Discussion

In this study, we demonstrated that post-CLP mice have impaired alveolar inflammation in response to intratracheal LPS. This post-septic “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,

393 an enzyme dedicated to the constitutive removal of 6-*O*-sulfo groups. We observed that
394 loss of Sulf-1 is necessary for impaired inflammation in post-septic animals, but it alone
395 was not sufficient to cause impaired inflammation in non-septic animals. Our data
396 therefore collectively indicate an endothelial role in CARS, a syndrome to date largely
397 relegated to post-septic leukocyte dysfunction.

398 Our findings indicate that sepsis suppresses endothelial Sulf-1, impairing
399 subsequent inflammatory responses after the resolution of septic endothelial injury.
400 Teleologically, this response may be designed to limit the degree of lung injury, shifting
401 endothelial signaling towards tissue repair. For example, an increase in 6-*O*-sulfation
402 promotes cell response to HS-binding growth factors, FGF2 and VEGF, which may
403 promote endothelial repair processes (6). However, this response may backfire if the
404 host is exposed to a secondary infection, by attenuating host-protective antimicrobial
405 responses. Post-septic patients are susceptible to secondary infection, with hospital-
406 acquired pneumonia the most common complication (15, 39). Septic patients who later
407 acquired secondary infections experience more severe illness, longer length-of-stay,
408 and higher 1-year mortality (39, 40). Therefore, in the presence of hospital-acquired
409 infection, loss of endothelial Sulf-1 may be detrimental. Additionally, we found that post-
410 septic animals had increased absolute neutrophil count in blood at the time of
411 intratracheal LPS (data not shown), indicating that decreased neutrophil infiltration
412 found in BAL fluid is not due to post-septic depletion of circulating neutrophils.
413 Interestingly, when the whole transcriptome profiles from circulating leukocytes were
414 compared between patients who acquired secondary infection and those who did not,
415 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 to 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 post-synthetic 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 pulmonary circulation are complex (13, 20), it has been reported that changes in sulfation in endothelial HS, particularly 6-*O*-sulfation,

439 weakens neutrophil binding to L-selectin and P-selectin (41, 42). Accordingly, the
440 immunosuppressive effects of loss of Sulf-1 may arise from numerous potential
441 processes, potentially converging upon pathways such as adhesion molecule
442 expression (**Figure 6**). Future studies will screen for 6-*O*-sulfated HS binding proteins,
443 providing greater insight into the downstream mechanisms responsible for our observed
444 effects of Sulf-1 on post-septic pulmonary inflammation.

445 An additional consideration is that changes in endothelial Sulf-1/2 expression
446 may impact the dynamics of glycocalyx degradation. Indeed, our endothelial microarray
447 experiments (GEO accession number: GSE129775) demonstrated that post-septic (48
448 h) loss of Sulf-1 coincided with increased expression of matrix metalloproteinases 8, 9,
449 25, and disintegrin and metalloproteinase domain-containing proteins 8, 15, 23. No
450 changes were seen in endothelial expression of heparanase or matrix metalloproteinase
451 15 in these post-septic mice. Future studies will be required to investigate the presence
452 of sulfatase-sheddase cross-talk and its relevance to lung injury and repair.

453 Sulf-1 and Sulf-2 preferentially targets trisulfated, NS2S6S disaccharides of HS,
454 and to a lesser extent, NS6S (22). Loss of Sulf-1, therefore, would be expected to
455 result in an increase of trisulfated, NS2S6S disaccharides (substrate), with a decrease
456 in NS2S (product). However, we rather observed an increase of HS overall 6-*O*-
457 sulfation (**Figure 2D**). This discrepancy may reflect the additional ability of Sulf-1 and
458 Sulf-2 to directly regulate HS biosynthesis enzymes (14). Therefore, it is possible that
459 our biological observations following Sulf-1 downregulation may be caused directly by
460 the loss of Sulf-1 activity, and/or indirectly, through a regulation of HS biosynthesis. Of
461 note, our recombinant Sulf-1 is of human origin. Based on the strong sequence

462 homology of HSulf-1 and MSulf-1, we do not expect any significant differences in
463 enzymatic activity (22), but we cannot exclude the possibility that enzymatic activity is
464 slightly different *in vivo*.

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

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Disclosure

None.

Figure Legends

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

Figure 2: The post-septic, reconstituted endothelial glycocalyx is remodeled. (A) At the 72 h time point characterized by pulmonary endothelial HS reconstitution, we injected 1 unit of heparinase I (Hep I) into the jugular vein, which degraded both sham and post-CLP pulmonary endothelial glycocalyx heparan sulfate. (B) In contrast, 1 unit of heparinase III (Hep III) into the jugular vein did not degrade post-septic pulmonary endothelial heparan sulfate. (C) Major sulfation positions on heparan sulfate constituent disaccharides. (D) Post-septic, reconstituted pulmonary endothelial heparan sulfate had a significantly increase ($P < 0.05$) in disaccharides with 6-*O*-sulfation (percentage of total sulfated disaccharides) as compared to heparan sulfate from sham animals. (E)

510 The percentage of 6-*O*-sulfated disaccharides increased over time in the plasma of
511 post-CLP mice. ESL: endothelial surface layer. n= 3-5 per group, Student's t-test, *$P <$
512 0.05.

513 **Figure 3: Post-septic pulmonary endothelial cells downregulate Sulf-1 mRNA**. (A)
514 We collected pulmonary endothelial cells and extracted total RNA at day 2, a time point
515 immediately prior to glycocalyx reconstitution at day 3. (B) Pulmonary endothelial cells
516 in mice 48 h after CLP had decreased Sulf-1 expression detected with RNA microarray.
517 (C) We validated the downregulation of Sulf-1 in pulmonary endothelial cells by qRT-
518 PCR using separate biological replicates. (D) We collected pulmonary endothelial cells
519 and extracted total RNA at 12 h after heparinase III injection, a time point immediately
520 prior to glycocalyx reconstitution after enzymatic, non-septic degradation. (E) mRNA
521 expression level of Sulf-1 in pulmonary endothelial cells were similar 12 h after
522 enzymatic degradation by heparinase III (Hep III) or heat-inactivated (HI) Hep III. n= 3-4
523 each group, Student's t-test, *$P < 0.05$.

524 **Figure 4: Loss of Sulf-1 contributes to post-septic CARS.** (A) Recombinant Sulf-1
525 was enzymatically active and efficiently removed 6-O sulfates from heparin, as shown
526 by the decrease in trisulfated [UA(2S)-GlcNS(6S)] disaccharide content (NS2S6S) and
527 concomitant increase of [UA-GlcNS(6S)] disulfated disaccharide (NS2S) in Sulf-1
528 treated heparin (white bars) compared to untreated control (black bars). (B) We
529 performed CLP on mice on day 0. On day 3, surviving post-septic mice were treated
530 with intravenous (IV) Sulf-1 (3 μg bolus) or diluent, then challenged with intratracheal
531 (IT) LPS (3 μg/g body weight). Animals were harvested 2 days after IT LPS. (C) Sulf-1
532 treated animals had increased leukocytes in BAL fluid 2 days after IT LPS, as compared

to 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 post-septic, diluent treated animal (stained with H&E) showed very little evidence of lung inflammation. (F) Lung histological section from a post-septic 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 μm and those on higher magnification images =100 μm. n= 4-5 each group, Student's t-test, $*P < 0.05$.

Figure 5: Loss of Sulf-1 is not sufficient to cause impaired inflammation in non-septic animals. (A) Sulf-$1^{f/f}$ Sulf-$2^{f/f}$ VEcadCreERT2$^{+ \text{ or } -}$ animals received tamoxifen or vehicle control injections intraperitoneally for 5 consecutive days (1 mg/day). Recombination of genes and pulmonary endothelial glycocalyx characteristics were evaluated 2 weeks after the last injection of tamoxifen (or vehicle). Knockout or control mice were alternatively challenged with IT LPS (3 μg/ g body weight) 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 non-endothelial cells (CD31-/CD144-), Lane 2: DNA from pulmonary endothelial cells (CD31+/CD144+). (C) Pulmonary endothelial glycocalyx of Sulf-1/2 knockout animals was resistant to heparinase III (Hep III) degradation, similar to the post-septic endothelial glycocalyx resistance to heparinase III observed in wild-type mice (**Figure 2B**). Control animals used were Sulf-$1^{f/f}$ Sulf-$2^{f/f}$ VEcadCreERT2^{-} (floxed gene alone without Cre recombinase), treated with tamoxifen. (D) Number of leukocytes in 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-$1^{f/f}$ Sulf-$2^{f/f}$ 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 μm and those on higher magnification images =100 μm. n = 3-6 each group, One-way ANOVA with post hoc Turkey test, *$P < 0.05$.

Figure 6: Knocking down 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 hours, which resulted in greater than 83% knockdown of Sulf-1. (B) Knock down of Sulf-1 resulted in downregulation of ICAM-1 expression in endothelial cells. Control cells were treated with lipofectamine without siRNA. n = 3, each data point represents average of 2-3 wells per treatment. Student's t-test, *$P < 0.05$.

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Figure 1 CARS model

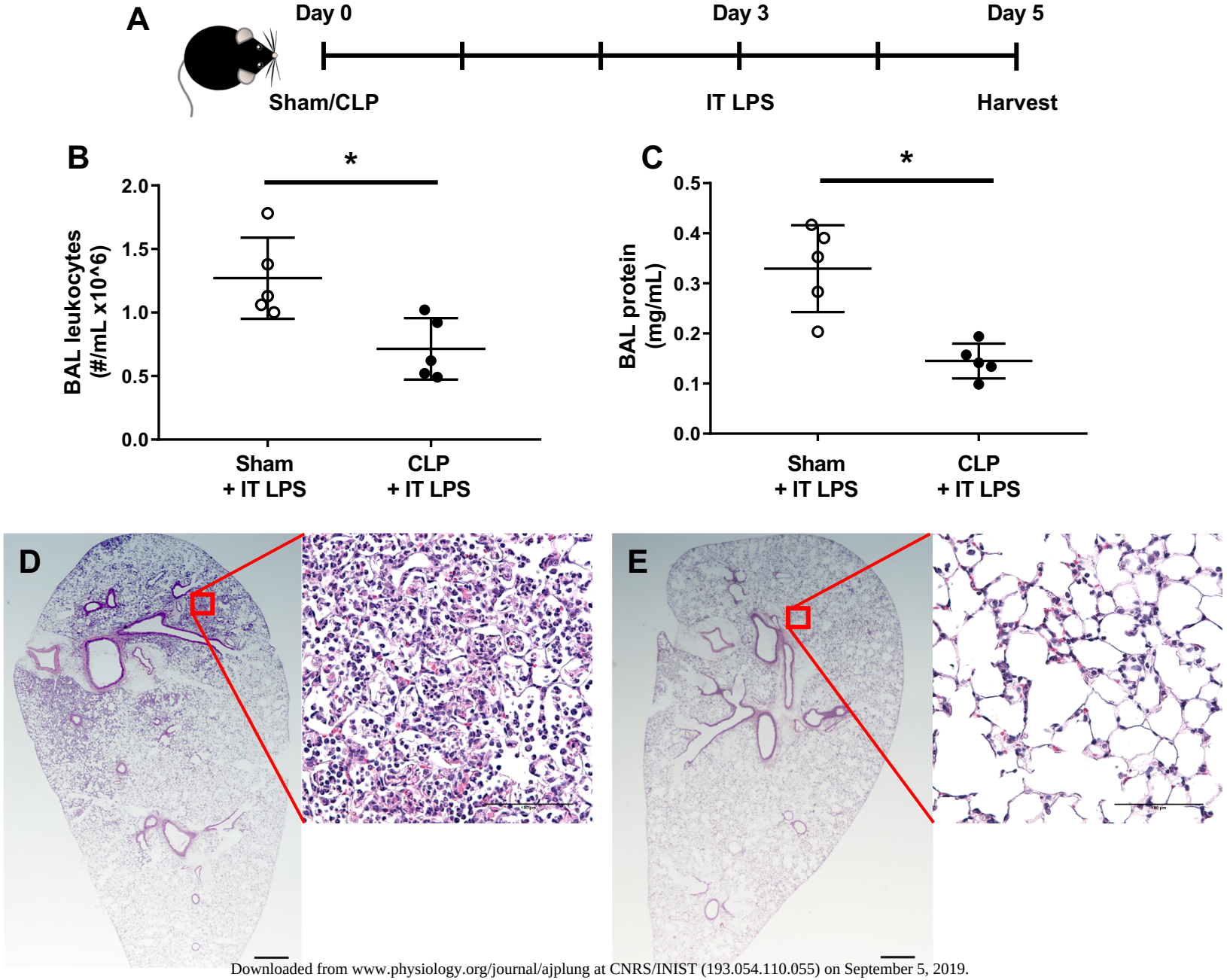


Figure 2 Post-septic, reconstituted glycocalyx acquires increased 6-O sulfation and newly found resistance against heparinase III digestion, but not against heparinase I

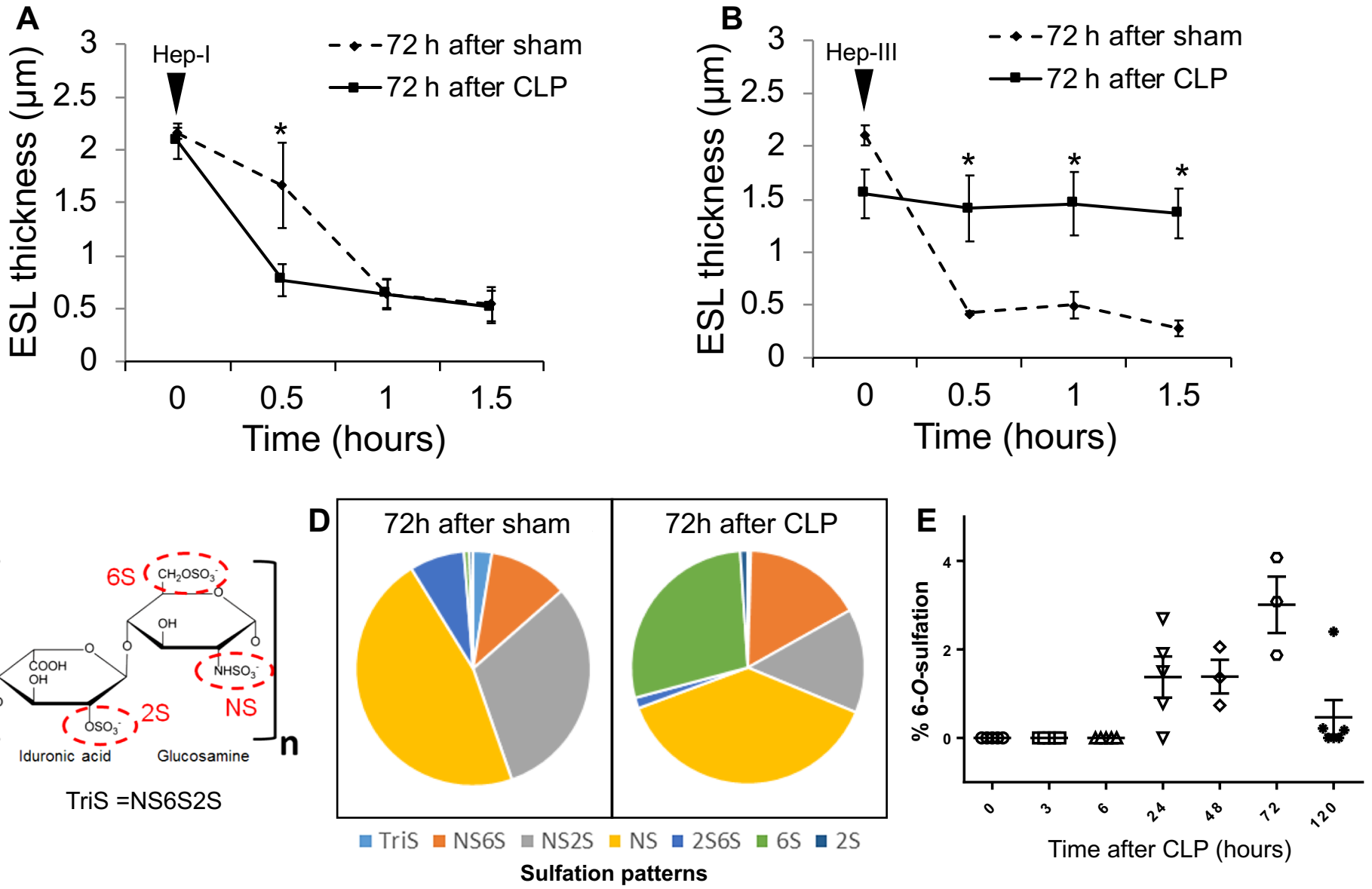


Figure 3 Post-septic pulmonary endothelial cells downregulate Sulf-1

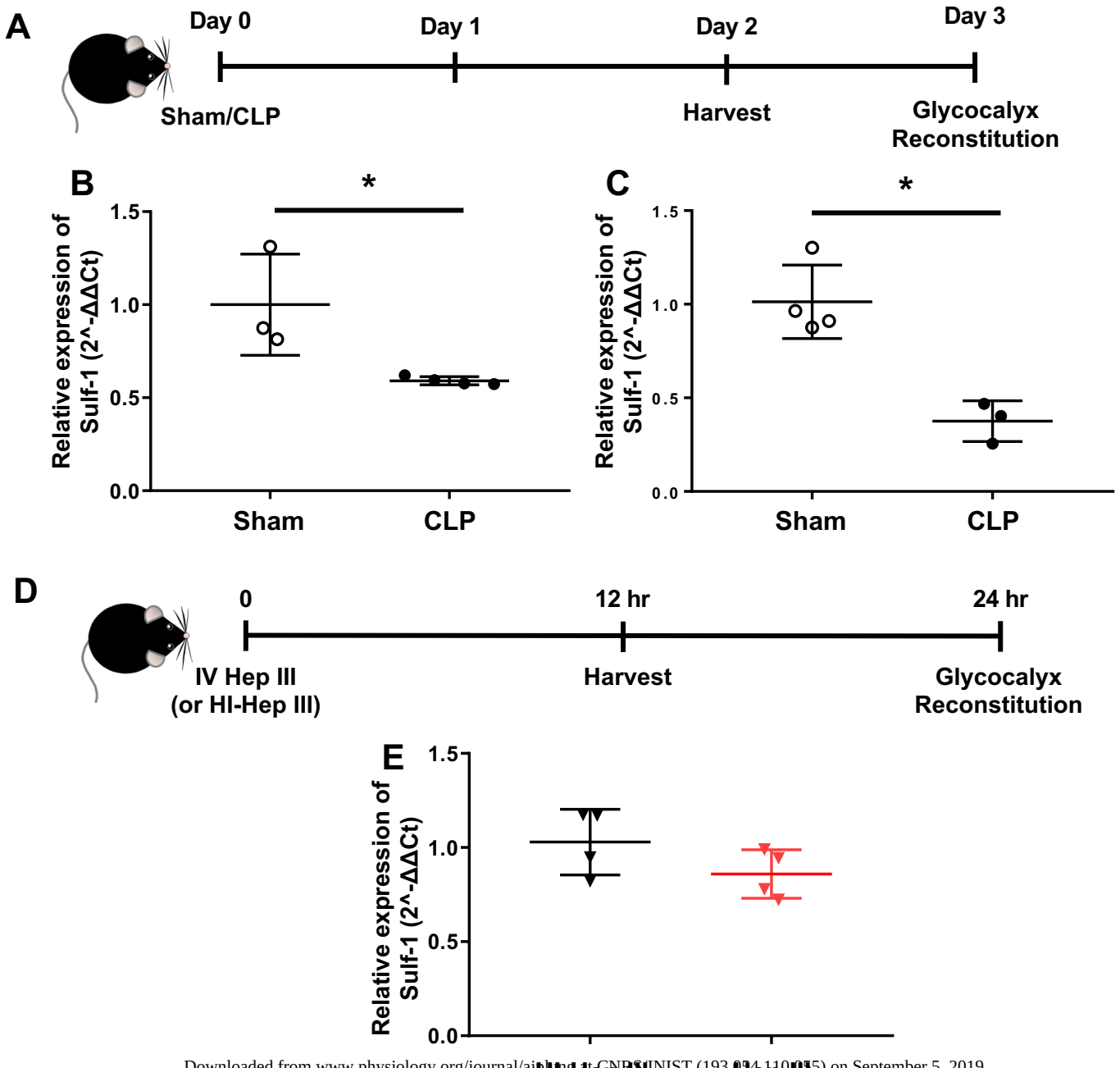


Figure 4 Sulf-1 is necessary for CARS

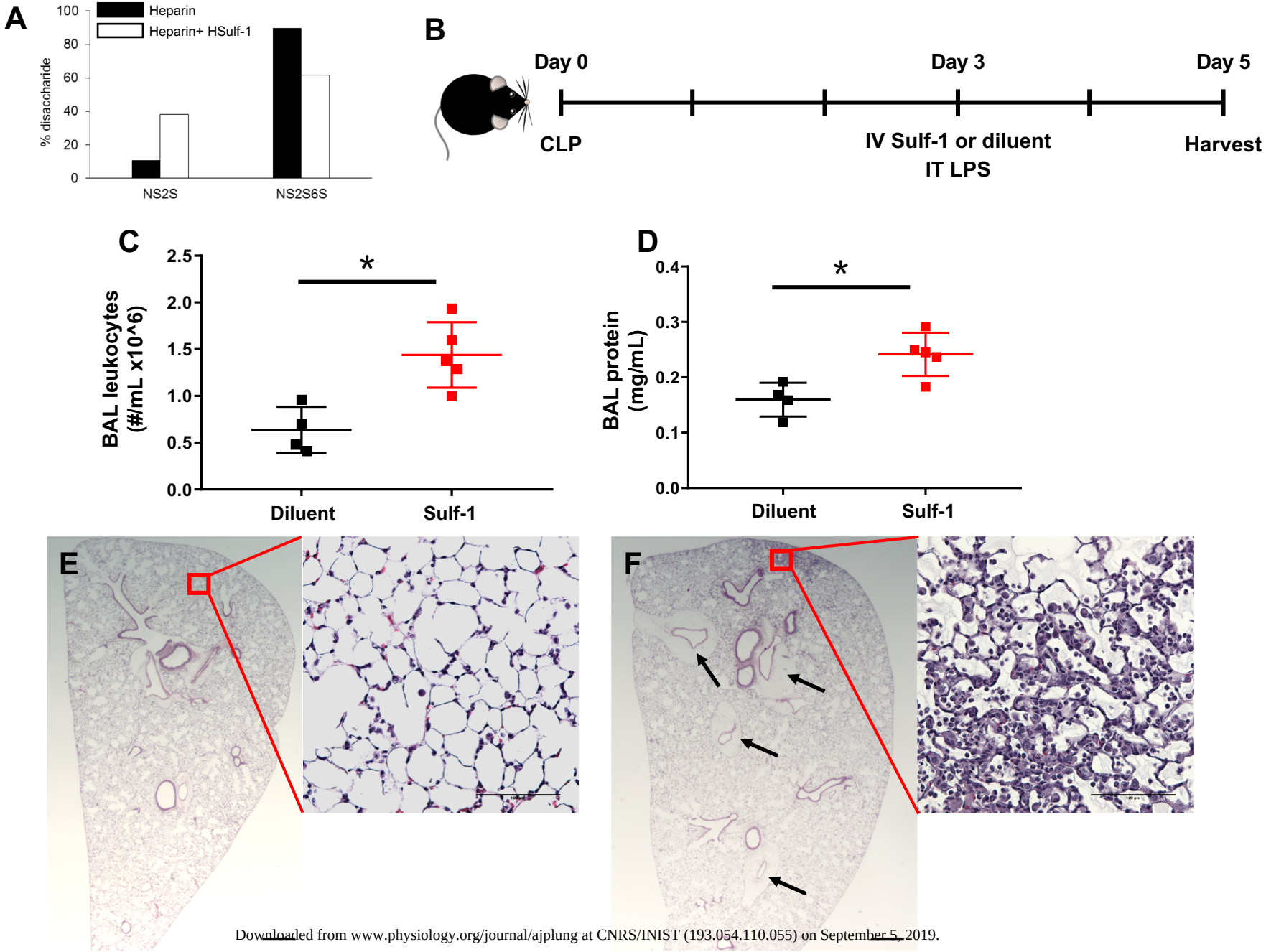
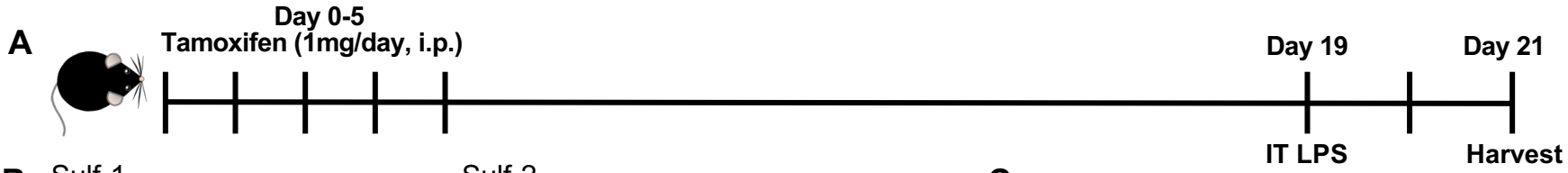
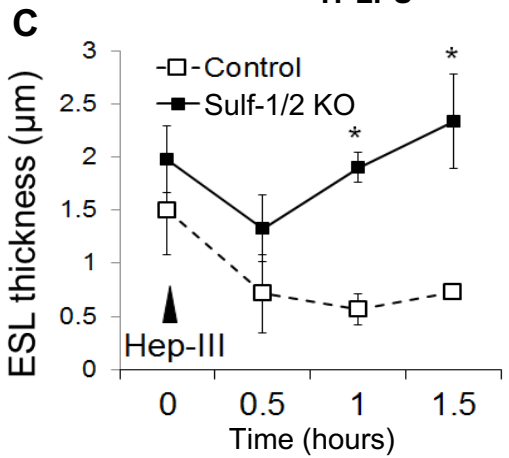
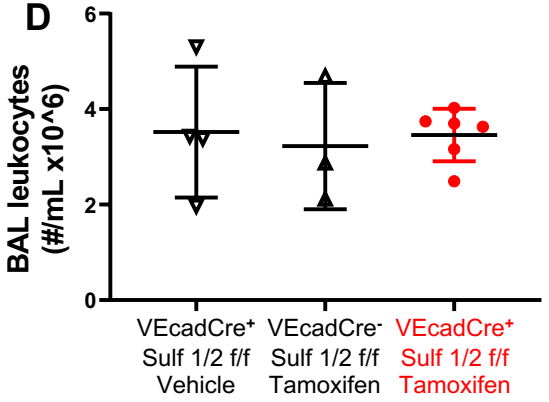


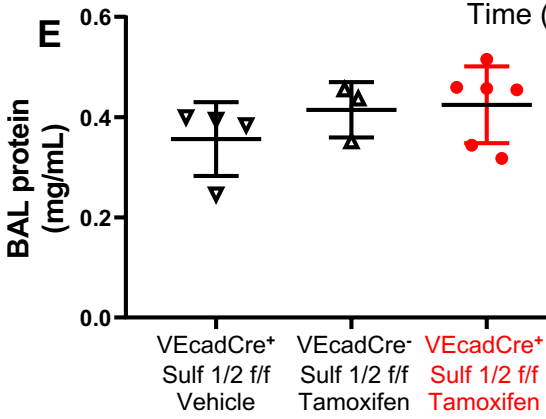
Figure 5 Loss of Sulf-1 is not sufficient to cause CARS in non-septic mice

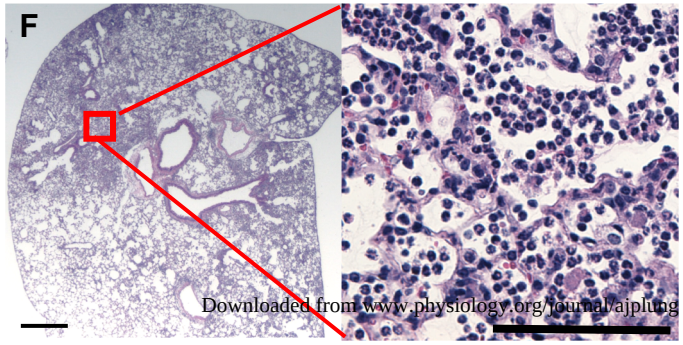




1: FAC sorted non-endothelial cells (CD31$^-$/CD144$^-$); **2**: FAC sorted endothelial cells (CD31$^+$/CD144$^+$)







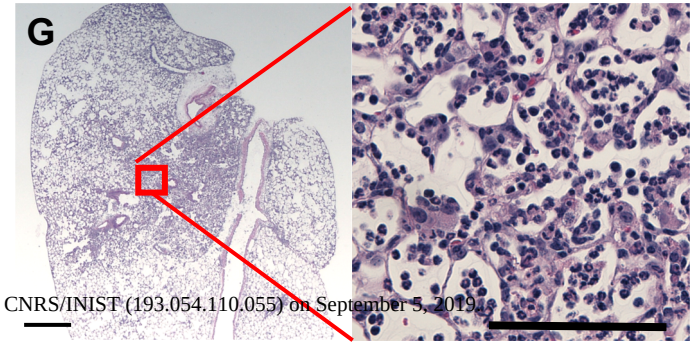


Figure 6 Knocking down Sulf-1 in pulmonary microvascular endothelial cells decreases ICAM-1 expression