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A disintegrin and metalloproteinase 15-mediated glycocalyx shedding contributes to vascular leakage during inflammation

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Aims Endothelial hyperpermeability exacerbates multiple organ damage during inflammation or infection. The endothelial glycocalyx, a protective matrix covering the luminal surface of endothelial cells (ECs), undergoes enzymatic shedding during inflammation, contributing to barrier hyperpermeability. A disintegrin and metalloproteinase 15 (ADAM15) is a sheddase capable of cleaving the ectodomains of membrane-bound molecules. Herein, we tested whether and how ADAM15 is involved in glycocalyx shedding and vascular leakage during sepsis.

Methods and results Dextran-150kD exclusion assay revealed lipopolysaccharide (LPS) significantly reduced glycocalyx thickness in mouse cremaster microvessels. Consistently, shedding products of glycocalyx constituents, including CD44 ectodomain, were detected with an increased plasma level after cecal ligation and puncture (CLP)-induced sepsis. The direct effects of CD44 ectodomain on endothelial barrier function were evaluated, which revealed CD44 ectodomain dose-dependently reduced transendothelial electrical resistance (TER) and caused cell–cell adherens junction disorganization. Furthermore, we examined the role of ADAM15 in CD44 cleavage and glycocalyx shedding. An *in vitro* cleavage assay coupled with liquid chromatography-tandem mass spectrometry confirmed ADAM15 cleaved CD44 at His^{235}-Thr^{236} bond. In ECs with ADAM15 knockdown, LPS-induced CD44 cleavage and TER reduction were greatly attenuated, whereas, ADAM15 overexpression exacerbated CD44 cleavage and TER response to LPS. Consistently, ADAM15 knockout in mice attenuated CLP-induced increase in plasma CD44. Intravital and electron microscopic images revealed ADAM15 deficiency prevented LPS-induced glycocalyx injury in cremaster and pulmonary microvasculatures. Functionally, $ADAM15^{-/-}$ mice with better-preserved glycocalyx exhibited resistance to LPS-induced vascular leakage, as evidenced by reduced albumin extravasation in pulmonary and mesenteric vessels. Importantly, in intact, functionally vital human lungs, perfusion of LPS induced a significant up-regulation of ADAM15, accompanied by elevated CD44 in the effluent and increased vascular permeability to albumin.

Conclusion Together, our data support the critical role of ADAM15 in mediating vascular barrier dysfunction during inflammation. Its mechanisms of action involve CD44 shedding and endothelial glycocalyx injury.

Keywords Endothelial barrier permeability • ADAM15 • Endothelial glycocalyx • CD44

1. Introduction

The endothelial glycocalyx covers the luminal surface of the vasculature and dynamically interacts with the endothelium and plasma constituents, regulating coagulation, leukocyte adhesion, and mechanotransduction. Emerging evidence has shown that the stability of the endothelial glycocalyx is essential to the maintenance of a selective permeability barrier of the vasculature.[1] Several experimental studies have demonstrated that the endothelial glycocalyx layer acts as an additional barrier to regulate the transvascular exchange of fluid and solutes.[2] Moreover, the classic

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Starling equation describing the movement of fluid across vascular barrier has been modified to take into account the contribution of the glycocalyx barrier on endothelial surface.[3]

The major components of the endothelial glycocalyx include proteoglycans, glycoproteins, and glycosaminoglycan side chains.[4] Proteoglycans, such as syndecans and glypicans, are regarded as the 'backbone' molecules of the glycocalyx, and are firmly connected to the endothelial cell (EC) membrane. Transmembrane glycoproteins, including CD44, provide further structural support for glycocalyx by creating more attachment points. The major glycosaminoglycans in the endothelial glycocalyx are heparan sulfate, chondroitin sulfate, and hyaluronic acid (HA), contributing to the viscoelastic properties of the endothelial glycocalyx. Heparan and chondroitin sulfate are covalently linked to the proteoglycan core proteins, whereas the non-sulfated glycosaminoglycan, HA, is attached to glycoprotein CD44. Glycocalyx degradation has been documented during inflammatory conditions, as evidenced by elevated plasma levels of glycocalyx injury markers, such as soluble CD44, HA, and heparan sulfate, correlating with the severity of inflammatory diseases.

CD44 is a transmembrane glycoprotein expressed on several cell types, including ECs, haematopoietic cells, and tumour cells. Functionally, CD44 interacts with various inter- and extracellular molecules and regulates a wide range of cell behaviours, including proliferation, cell-matrix adhesion, cell–cell interactions, and intracellular signal transduction.[5] CD44 expressed on vascular ECs binds to its primary ligand HA, thereby anchoring glycocalyx to the EC surface. Furthermore, several lines of evidence indicate the role of CD44 in maintaining the integrity of the microvascular barrier. Mice lacking CD44 display impaired barrier function,[6] and CD44 knockout ECs are more permeable to small molecules.[7] Similar to other glycocalyx components, CD44 has also been reported to undergo enzymatic cleavage, resulting in elevated soluble form of CD44 in the circulation.[8] Given the important roles of CD44 in glycocalyx integrity and vascular barrier function, it is plausible that preventing CD44 cleavage may confer protection against glycocalyx destruction and vascular hyperpermeability caused by inflammatory injury, including sepsis.

Metalloproteinases catalyse the hydrolysis of proteins with the aid of metal ions in their active site. Despite several studies showing that matrix metalloproteinase-9 (MMP-9)[9] and membrane-type 1 MMP[8] are capable of cleaving CD44 in tumour cells, their effects on the endothelial glycocalyx barrier under the particular condition of septic injury have not been established. A disintegrin and metalloproteinase 15 (ADAM15) belongs to a family of membrane-bound metzincin metalloproteinases. The metalloproteinase domain of ADAM15, located in the extracellular region, accounts for its proteinase activity and enables it to enzymatically cleave E-cadherin, N-cadherin, transforming growth factors, etc.[10] Functionally, ADAM15 has been implicated in several pathophysiological conditions, including rheumatoid arthritis and metastatic progression of different cancers. Of note, our previous studies revealed the involvement of ADAM15 in vascular inflammation. We suggested that up-regulation of ADAM15 contributes to lipopolysaccharide (LPS)-induced lung injury and endothelial barrier dysfunction.[11,12]

In this study, we sought to determine if ADAM15 causes glycocalyx injury by cleaving CD44, and whether its shedding products have any direct signalling effects on endothelial barrier function during sepsis. Our findings from experiments utilizing recombinant proteins, cultured endothelial monolayers, animal models of sepsis, and *ex vivo* human lungs indicate that ADAM15 is capable of cleaving CD44 at the membrane-proximal region of CD44; deficiency of ADAM15 blocks sepsis-stimulated CD44 cleavage and glycocalyx thinning. More importantly, ADAM15$^{-/-}$ mice with more intact glycocalyx on microvascular endothelium display less vascular leakage following septic injury, compared with wild-type mice. Our work sheds light on the new role of ADAM15 in glycocalyx degradation, suggesting ADAM15 as a promising target for preventing vascular leakage in sepsis.

2. Methods

For details, see Supplementary material online.

2.1 Animals

ADAM15$^{-/-}$ mice with a C57/BL6 background were generated based on breeding pairs generously provided by Dr Carl P. Blobel (Hospital for Special Surgery, New York, NY, USA).[13] ADAM15$^{-/-}$ mice and wild-type controls were randomly assigned into experimental groups. All animal experiments were performed conform to the NIH Guide for the Care and Use of Laboratory Animals and approved by the University of South Florida Institutional Animal Care and Use Committee. The information regarding the animal strain, age, and sex is listed in Supplementary material online, *Table S1*.

2.2 Reagents

Detailed information (company and catalogue number) regarding the reagents used in this study is listed in Supplementary material online, *Table S2*.

2.3 Intravital microscopic measurements of glycocalyx thickness and FITC-albumin transvascular flux

Mice were anesthetized (Urethane, 1.75×10^3 mg/kg) 24 h after LPS (10 mg/kg, i.p.) or vehicle control injection. A jugular vein cannulation was performed to infuse FITC-dextran (150 kD, 100 mg/kg) or FITC-albumin (a bolus of 100 mg/kg followed by a continuous infusion of 0.15 mg/kg/min) for observation of cremaster or mesenteric microvessels, respectively, using intravital microscopy (Nikon Eclipse E600FN, Tokyo, Japan). The bright-field and fluorescent images were captured via Evolve 512 digital camera (Photometrics, AZ, USA), and analysed with ImageJ (v1.48i; NIH, USA). Cremaster microvascular glycocalyx thickness was calculated as one half of the difference between anatomical (bright-field) and fluorescent vascular width.[14] The fluorescence intensity inside (Ii) and intensity outside (Io) a selected mesenteric post-capillary venule was used to measure the transvascular flux of FITC-albumin, determined by [1- (Ii-Io)/Ii].[15]

2.4 Evans blue-bound albumin extravasation

Fifty mg/kg Evans Blue was injected intravenously to anesthetized mice and allowed to circulate for 30 min. Blood was then flushed out with Lactated Ringer's (LR) solution via transcardial perfusion. The left lobe of mouse lung was removed and imaged at 700 nm infrared channel using LI-COR imaging system (Odyssey CLx; USA) to visualize the extravasated Evans Blue-bound albumin.[15]

2.5 Electron microscopic observation of pulmonary microvascular glycocalyx

Animals were anesthetized 24 h after LPS or control treatment. Blood was removed by transcardial perfusion with LR solution containing 4%

bovine serum albumin (BSA). Mice were perfusion-fixed and stained for the glycocalyx with fixative solution (0.1M sodium cacodylate, 4.375% glutaraldehyde, and 0.1% lanthanum) containing 0.075% alcian blue for 30 min at room temperature. Lung tissue was excised, trimmed into 1 mm^3 segments, and placed in phosphate-buffered 0.1 M sodium cacodylate. Post-fixation, embedding, and sectioning of lung segments were performed by the Lisa Muma Weitz Microscopy Core Laboratory. Images were captured by transmission electron microscope (JEOL1400, Tokyo, Japan).[16]

2.6 CLP

The cecum was exposed from anesthetized mice, tightly ligated at 5 mm below the ileocecal valve and perforated twice with a 20-gauge needle distal to the point of ligation. One mm of faecal matter was extruded from each puncture hole. The cecum was then repositioned inside the abdomen, and the abdominal cavity was closed in two layers. Mice then received LR solution for resuscitation. Sham controls were subjected to the same surgical procedures but without ligation and puncture.

2.7 *In vitro* cleavage assay and proteomic analysis

Ten μL of full-length recombinant CD44 (≥0.05 mg/mL) was mixed with 1, 3, or 10 μL of ADAM15 (0.5 mg/mL) and incubated in 37°C for 6 h on a rotator. The mixture was then separated via SDS-PAGE and immunoblotted for CD44. To identify the cleavage site, the cleaved CD44 band was excised and sent to the Proteomics Core in Moffit Cancer Center and Weill Cornell Medicine Proteomics and Metabolomics Core Facility. The cleaved CD44 band was digested by trypsin and sequenced via LC-MS/MS. Non-R/K ending peptides (non-tryptic peptides) repeatedly identified by both independent proteomic analyses were considered produced by ADAM15 cleavage, and the c-terminal amino acid of this non-R/K ending peptide was the cleavage site.

2.8 Transendothelial electrical resistance

Endothelial barrier function was determined using an electric cell-substrate impedance sensing (ECIS) system (Applied Biophysics, Troy, NY, USA). Human umbilical vein endothelial cells (HUVECs) were seeded on ECIS electrode arrays and cultured into confluent monolayers using complete EGM-2 medium. One μg/mL LPS or vehicle control was applied to induce inflammation. As the indicator for endothelial cell–cell barrier integrity, real-time changes in transendothelial electrical resistance (TER) were recorded and analysed.

2.9 siRNA transfection and gene overexpression

Transfections of siRNA and plasmid of ADAM15 were conducted using 4D-Nucleofector™ systems (Lonza, Switzerland) as previously described.[15] Briefly, HUVECs were cultured to 90% confluence, trypsinized, pelleted, and resuspended with 100 μL of P5 primary cell 4D-Nucleofector™ solution containing 1 μM siRNA or 2 μg plasmid. After electroporated using CA-167 program, ECs were plated onto culture dishes or ECIS arrays for further experiments.

2.10 Human lung *ex vivo* perfusion assay

Human lungs donated through the LifeLink Foundation were used for *ex vivo* perfusion assay. The lungs were acquired from brain-dead donors after consent was obtained from next of kin in accordance with Florida State Statutes and The Declaration of Helsinki. At the hospital, the lungs were removed *en bloc*, partially inflated, closed at the trachea, and placed on ice for transportation. Upon arrival, the pulmonary vessels were cannulated with tubing (Tygon E-3603) to create a circulating system. The trachea was cannulated with endotracheal tube (10.0, SunMed), and the lungs were ventilated via Hamilton C1 ventilator (Hamilton Medical, Switzerland) with a tidal volume of 7 mL/kg of body weight, positive end expiratory pressure of 5 cm H_2O, and respiratory rate of 7 breath/min, using 21% oxygen.[17] The lungs were then perfused with ~37°C Dulbecco's Modified Eagle's medium containing 5% BSA with or without 0.25 mg/mL LPS for 6 h.[18] After perfusion, lung tissue and the perfusion effluent were collected for further analyses.

2.11 Enzyme-linked immunosorbent assay

Mouse blood was collected by cardiac puncture using a heparinized syringe and centrifuged at 2000×*g* for 15 min to isolate the plasma. The EC conditioned media and human lung perfusion effluent were centrifuged to remove dead cells and debris. The level of HA and CD44 were measured with enzyme-linked immunosorbent assay (ELISA) Kits per manufacturer's instructions.

2.12 Statistical analysis

All data meet normal distribution assumption. Statistical analyses were performed via GraphPad Prism (6.0g). The statistical tests used for all data analyses are listed in Supplementary material online, *Table S3*.

3. Results

3.1 LPS induces glycocalyx degradation and microvascular barrier disruption

To measure the glycocalyx thickness in mouse cremaster microvessels, we applied intravital microscopy combined with intravenous administration of fluorescein isothiocyanate-dextran (FITC-dextran) (150 kDa). Post-capillary venules were selected for microscopic observation, and the diameters of these microvessels were comparable between groups (Supplementary material online, *Figure S1*). Given the nature of the endothelial glycocalyx to exclude large molecules from the vessel wall, the difference in vascular widths under bright-field and fluorescence represents the thickness of the glycocalyx layer.[14] As shown in *Figure 1A* and *B*, the average glycocalyx thickness in mouse cremaster microvessels is around 0.8 μm under normal conditions. However, mice subjected to LPS-induced septic injury displayed a prominent reduction in the glycocalyx thickness 24 h after the induction of sepsis.

As a biomarker of glycocalyx injury, the circulating HA level in mouse plasma was measured to confirm glycocalyx degradation. Consistent with intravital microscopic results, a very low level of circulating HA was detected in the sham group, although mice receiving LPS injection exhibited a drastically increased level of plasma HA (*Figure 1C*). In addition, we determined the pulmonary vascular barrier integrity by measuring the extravasation of Evans Blue, a dye with high affinity for serum albumin. Infrared images in *Figure 1D* showed that LPS treatment led to a significant increase in Evans Blue-bound albumin leakage into the extravascular space in mouse lungs, indicating disrupted pulmonary vascular barrier.

3.2 CD44 cleavage and effects on endothelial barrier permeability

Endothelial CD44 functions as a primary HA receptor, which ensures the attachment of HA, an important glycocalyx constituent, on EC surface. The elevated plasma level of HA may result from the impaired

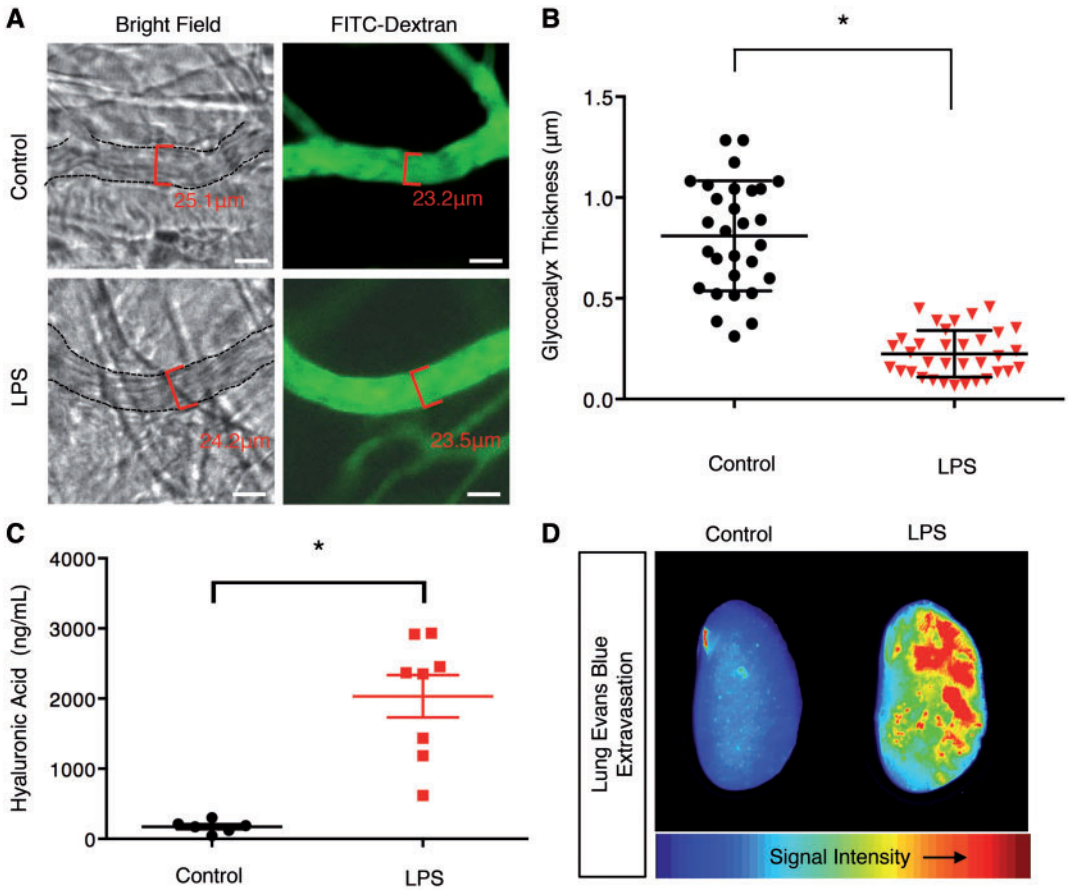

Figure 1 LPS causes glycocalyx shedding and microvascular leakage. (*A*, *B*) Measurement of mouse microvascular glycocalyx thickness. Mouse cremaster microvascular glycocalyx was determined using FITC-dextran (150 kD) exclusion assay 24 h after LPS injection. (*A*) Representative bright-field and fluorescent images of mouse cremaster microvessels. Scale bar = 20 μm. (*B*) Quantification of the glycocalyx thickness (mean ± standard deviation). $n = 5$ independent animals in each group, each dot represents the average of three measurements in one microvessel. $^*P < 0.05$. (*C*) The effects of LPS on the plasma level of HA. Mouse plasma was collected 24 h after LPS injection (mean ± standard error of the mean (SEM), $n = 6$). $^*P < 0.05$. (*D*) Evans Blue extravasation showing plasma protein leakage in mouse lung. Left lung lobes from vehicle or LPS-treated mice were scanned with infrared imaging system. Signal intensity is proportional to the amount of Evans Blue-bound albumin extravasated into the extravascular space. $n = 4$. $^*P < 0.05$.

structural and functional integrity of endothelial CD44. Indeed, as shown in *Figure 2A*, *a* significantly increased plasma level of soluble CD44 was detected in mice subjected to cecal ligation and puncture (CLP)-induced septic injury, demonstrating that sepsis enhanced the ectodomain shedding of CD44.

Next, we sought to determine the direct effect of the CD44 cleavage product-its ectodomain, on endothelial barrier permeability. Recombinant CD44 ectodomain was applied to confluent HUVEC monolayers. In *Figure 2B*, real-time TER recordings, an indicator of endothelial barrier integrity, demonstrated that the ectodomain of CD44 led to endothelial barrier dysfunction in a dose-dependent manner. Given that the barrier function of ECs is primarily dependent on adherens junctions, we then tested the morphological changes in VE-cadherin and β-catenin upon CD44 ectodomain treatment. Immunofluorescence imaging showed intense and continuous staining of VE-cadherin and β-catenin at cell–cell contacts of vehicle-treated ECs; however, in response to the administration of CD44 ectodomain, these junction proteins became dissociated and disrupted along EC borders (*Figure 2C*). Consistently, quantitative analysis confirmed that the ectodomain of CD44 significantly raised the level of adherens junction discontinuity (*Figure 2D*). We further investigated the molecular mechanism underlying CD44 ectodomain-induced junction disruption. A western blot analysis (*Figure 2E* and *F*) indicated that CD44 ectodomain mediated an increase in tyrosine phosphorylation of VE-cadherin, a well-known signalling event that triggers the dissociation of VE-cadherin-β-catenin complex and destabilizes the junction structure at cell–cell contacts. Moreover, treatment of ECs with CD44 ectodomain enhanced N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced neutrophil transmigration across EC monolayer, which has been commonly used as an indicator of barrier dysfunction (Supplementary material online, *Figure S2*).

3.3 ADAM15 cleaves CD44 at membrane-proximal region

These intriguing results with the CD44 ectodomain prompted us to investigate the proteinase(s) responsible for CD44 cleavage. ADAM15, containing zinc-binding metalloproteinase, disintegrin, cysteine-rich, and EGF-like domains, has been well characterized for its proteolytic

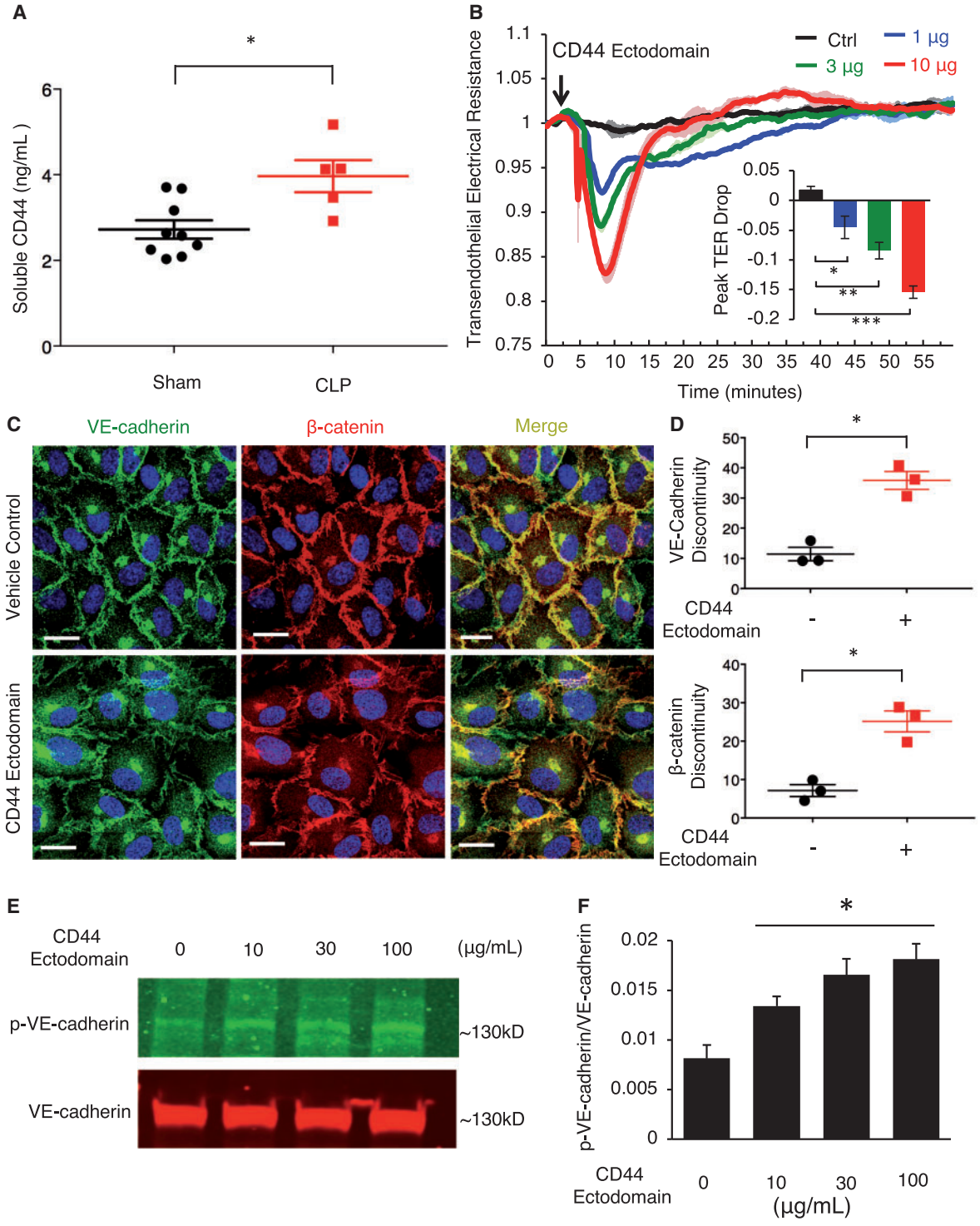

Figure 2 CD44 ectodomain exhibits barrier disruptive effects. (*A*) The plasma level of soluble CD44 in mice subjected to sham or CLP induced-sepsis (mean ± SEM, $n = 5$). (*B*) Time- and does-dependent TER tracing showing the effects of CD44 ectodomain on endothelial barrier function. Values were normalized to their baseline ($t = 0$). Solid line represents the mean resistance, and shadow represents standard error. Embedded bar graph indicates maximal TER drop (mean ± SEM, $n = 3$). (*C*) Representative confocal images of endothelial adherens junctions upon vehicle or CD44 ectodomain (10 μg) treatment. Scale bar = 20 μm. (*D*) Discontinuity of VE-cadherin and β-catenin at cell–cell junctions. The number of discontinuous adherens junction positive-stained regions was quantified using Imaris software (mean ± SEM, $n = 3$). Each dot represents the average value from five fields of view. (*E*, *F*) The effects of CD44 ectodomain on tyrosine phosphorylation of VE-cadherin. CD44 ectodomain was applied to HUVEC monolayer and incubated for 5 min. Cell lysate was immunoblotted for phosphorylated VE-cadherin (p-VE-cadherin) and total VE-cadherin (VE-cadherin). (*E*) Representative western blot images. (*F*) The ratio of densitometry value of p-VE-cadherin to total VE-cadherin (mean ± SEM, $n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. vehicle control.

activities. Our immunofluorescence images showed evidence for co-localization of ADAM15 and CD44 on HUVEC membrane (Supplementary material online, *Figure S3*), suggesting potential interactions between the two molecules. To test whether ADAM15 catalyses CD44 cleavage, we conducted an *in vitro* cleavage assay by incubating recombinant ADAM15 with CD44 (N-terminal GST-tagged), followed by immunoblot analysis of the cleavage products. As shown in *Figure 3A*, full-length CD44 appeared at ~70kD; meanwhile, at lanes where ADAM15 was applied, a cleaved CD44 band below the full-length band was detected. Densitometry analysis indicated that the intensity of full-length CD44 was inversely related to the amount of ADAM15 applied, and the cleaved CD44 was produced by ADAM15 cleavage in a dose-dependent manner (*Figure 3B*). We then re-probed this blot for N-terminal GST tag and the positive GST staining in the cleaved CD44 band indicated the cleavage site was outside of the N-terminal region (Supplementary material online, *Figure S4*).

In an effort to further identify the potential cleavage site, the cleaved CD44 band was digested by trypsin and analysed via liquid chromatography-tandem mass spectrometry (LC-MS/MS). Digested peptides identified by LC-MS/MS (*Figure 3C*, shown in red) were matched to the full-length CD44 protein sequence. As trypsin exclusively catalyzes the hydrolysis of the carboxyl side of arginine (R) and lysine (K), any identified peptides with a C-terminal amino acid other than R or K were produced by ADAM15-mediated cleavage and that C-terminal amino acid was considered the ADAM15 cleavage site within CD44. Our results indicated that the cleavage site was His^{235}, which falls in the membrane-proximal region of CD44 extracellular domain.

3.4 CD44 cleavage is regulated by ADAM15 expression and correlated with endothelial barrier disruption

ADAM15-mediated CD44 cleavage was further verified in HUVECs via manipulating ADAM15 gene expression. LPS treatment induced notably increased ADAM15 expression on the EC membrane and enhanced soluble CD44 in EC conditioned medium (*Figure 4A* and *B*). To confirm that LPS-triggered CD44 cleavage is mediated by ADAM15, we silenced ADAM15 expression with siRNA. Western blot results in *Figure 4A* confirmed the up-regulation of ADAM15 caused by LPS treatment was inhibited by ADAM15 knockdown. Furthermore, LPS-induced increased soluble CD44 in conditioned medium was significantly blocked by ADAM15 knockdown, indicating reduced CD44 cleavage in ADAM15 knockdown ECs (*Figure 4B*). More importantly, real-time TER tracing showed that ADAM15 knockdown ECs with less CD44 cleavage appeared to exhibit attenuated TER reduction in response to LPS challenge, compared with scrambled siRNA-treated ECs (Supplementary material online, *Figure S5A* and *B*).

Next, we overexpressed ADAM15 in HUVECs and assessed its impacts on CD44 cleavage and endothelial barrier function. The efficacy of ADAM15 overexpression was confirmed by western blot (*Figure 4C*). Our data demonstrated that augmented CD44 cleavage occurred in ADAM15 overexpressed ECs (*Figure 4D*). Meanwhile, ADAM15 overexpression exacerbated LPS-induced reduction in endothelial barrier resistance (Supplementary material online, *Figure S5C* and *D*). These results demonstrated that CD44 cleavage was mediated by ADAM15 and closely associated with endothelial barrier dysfunction.

3.5 ADAM15$^{-/-}$ mice are resistant to glycocalyx degradation and vascular leakage during sepsis

To further validate our results, we turned our focus to *in vivo* studies utilizing ADAM15 deficient mice. Previous characterization revealed that loss of ADAM15 in mice does not cause obvious developmental defects or pathological phenotypes.[13] Moreover, we found that the glycocalyx thickness is comparable between wild-type and ADAM15$^{-/-}$ mice under normal conditions (data not shown). However, ADAM15 deficiency significantly attenuated sepsis-induced CD44 ectodomain shedding, as evidenced by reduced plasma level of soluble CD44 in ADAM15$^{-/-}$ mice compared with wild-type controls (*Figure 5A*). Consistently, intravital microscopic data in *Figure 5B* and *C* showed that ADAM15 knockout remarkably prevented LPS-stimulated endothelial glycocalyx thinning in mouse cremaster vessels. Moreover, transmission electron microscopy (TEM) images of the endothelial glycocalyx in mouse pulmonary microvessels revealed direct evidence showing that mice lacking ADAM15 were resistant to LPS-induced reduction in endothelial glycocalyx coverage (*Figure 5D*).

Furthermore, we compared the barrier permeability of various microvasculatures in wild-type and ADAM15$^{-/-}$ mice after LPS challenge. In mouse lungs, knockout of ADAM15 significantly blocked LPS-induced Evans Blue-bound albumin leakage into the extravascular space (*Figure 5E*). We also determined the barrier integrity of mouse mesenteric vessels by measuring the transvascular flux of FITC-albumin. In healthy mice, FITC-albumin remained in intravascular space, indicating an intact vascular barrier. However, mice receiving LPS treatment displayed a massive accumulation of FITC-albumin in the interstitial compartment, which was significantly ameliorated in ADAM15$^{-/-}$ mice (*Figure 5F*).

3.6 Human lungs receiving LPS display up-regulated ADAM15 expression and disrupted integrity of the endothelial glycocalyx and vascular barrier

Our *in vitro* and *in vivo* findings were further tested with human organs. Non-transplantable donated human lungs were ventilated and perfused with LPS using an *ex vivo* perfusion system established in our lab (*Figure 6A*). Western blot data in *Figure 6B* indicated LPS treatment greatly up-regulated the expression of ADAM15 in the perfused human lung tissue. Meanwhile, soluble glycocalyx components in the lung perfusion effluent were detected to assess the extent of glycocalyx injury. Consistently, LPS stimulation drastically increased soluble CD44 level in the perfusion effluent (*Figure 6C*); a similar trend was also found for the level of HA (*Figure 6D*), indicating enhanced glycocalyx damage after LPS treatment. We then determined the vascular barrier function of perfused human lungs. In comparison to control lungs, LPS-treated lungs exhibited increased permeability to Evans Blue-bound albumin (*Figure 6E*).

4. Discussion

This study reports a novel role of ADAM15 in regulating endothelial glycocalyx integrity and barrier function. Our data demonstrates: (i) ADAM15 is capable of cleaving the glycocalyx protein CD44 at the membrane proximal region and releasing its ectodomain into the circulation; (ii) CD44 ectodomain interacts with ECs causing barrier dysfunction; (iii) ADAM15 deficiency blocks sepsis-induced reduction in the thickness and coverage of endothelial glycocalyx in mouse microvessels;

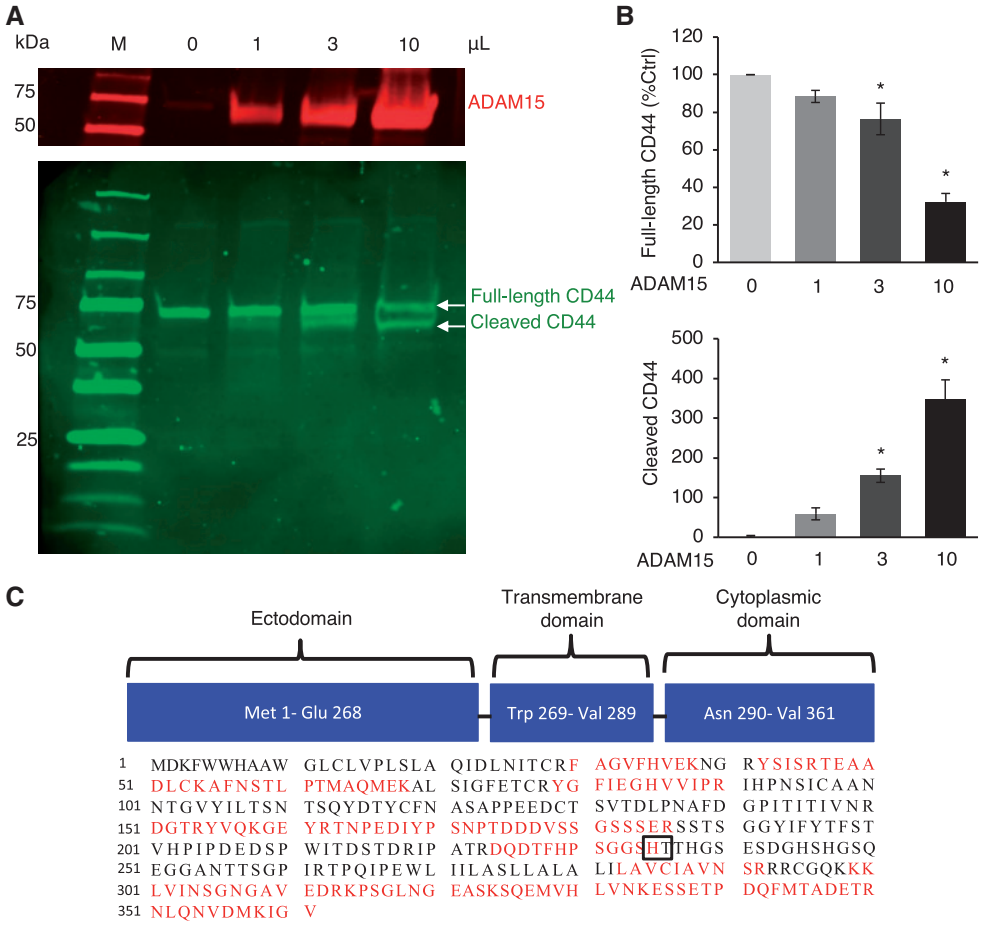

Figure 3 Recombinant ADAM15 cleaves CD44 at His^{235}-Thr^{236} bond. (*A*) Western blot showing ADAM15 cleaves CD44 in a dose-dependent manner. Recombinant ADAM15 was incubated with CD44 at 37°C for 6 h. The protein mixture was immunoblotted for ADAM15 and CD44. (*B*) Densitometry analysis of full-length CD44 and cleaved CD44 bands (mean ± SEM, $n = 3$). *$P < 0.05$ vs. control. (*C*) Schematic diagram of the CD44 amino acid sequence and cleavage site. Cleaved-CD44 band was digested by trypsin, sequenced via LC-MS/MS and matched with the full-length CD44 sequence (matched amino acids were in red). The C-terminal amino acid His^{235} (in black box) of the non-tryptic peptide was considered the cleavage site.

(iv) ADAM15-mediated glycocalyx injury contributes to vascular hyperpermeability during inflammation. To the best of our knowledge, these findings provide the first line of direct evidence for ADAM15-induced CD44 cleavage and glycocalyx shedding during septic injury. As diagrammed in *Figure 7*, we hypothesize that ADAM15 shedding CD44 has three-fold impact on endothelial barrier dysfunction. First, cleavage of core glycoproteins results in the loss of attachment sites for glycosaminoglycans network and disrupts the structural integrity of glycocalyx, impairing its barrier property against macromolecular leakage. Second, reduced glycocalyx coverage increases the exposure of endothelium to inflammatory mediators and activated leukocytes in the circulation. Thirdly, the cleaved products, CD44 ectodomains, act as inflammatory signals causing barrier dysfunction. Thus, inhibition of ADAM15 expression or blocking its function may confer protection of the structural and functional integrity of microvascular endothelial barriers.

Endothelial barrier dysfunction has been well recognized as a critical component of inflammatory response to infection, injury, and ischemic diseases. Leakage of plasma proteins and fluid into the extravascular space leads to reduced blood volume while increasing interstitial pressure, resulting in poor organ perfusion. Meanwhile, excessive fluid accumulating in the extravascular space impairs blood-tissue gas exchange, along with inadequate tissue perfusion, contributing to multiple organ dysfunction.[19,20] This study highlights the importance of maintaining the endothelial glycocalyx structural integrity in preventing vascular barrier leakage during sepsis. Since, the first electron microscopy image showing the existence of the endothelial glycocalyx in 1966,[21] our understanding of its structure and function has advanced. The barrier property of the glycocalyx layer on endothelial surface was first indicated in 1990s by Noble *et al.*[22] and Adamson *et al.*[23] The role of the endothelial glycocalyx in vascular barrier regulation is further supported by the study showing that glycocalyx digestion mediated by angiopoietin-2 increases vascular permeability in mice.[24] Moreover, the 'double-barrier concept' introduced by Rehm *et al.*[2] recognizes glycocalyx as an additional barrier controlling the out-flow of fluid and solutes in coronary vasculature. In line with these findings, our data showed that septic mice with impaired glycocalyx layer exhibited hyperpermeability in pulmonary and mesenteric microvessels. In contrast, microvessels from $ADAM15^{-/-}$ mice with preserved glycocalyx structure displayed attenuated leak response to

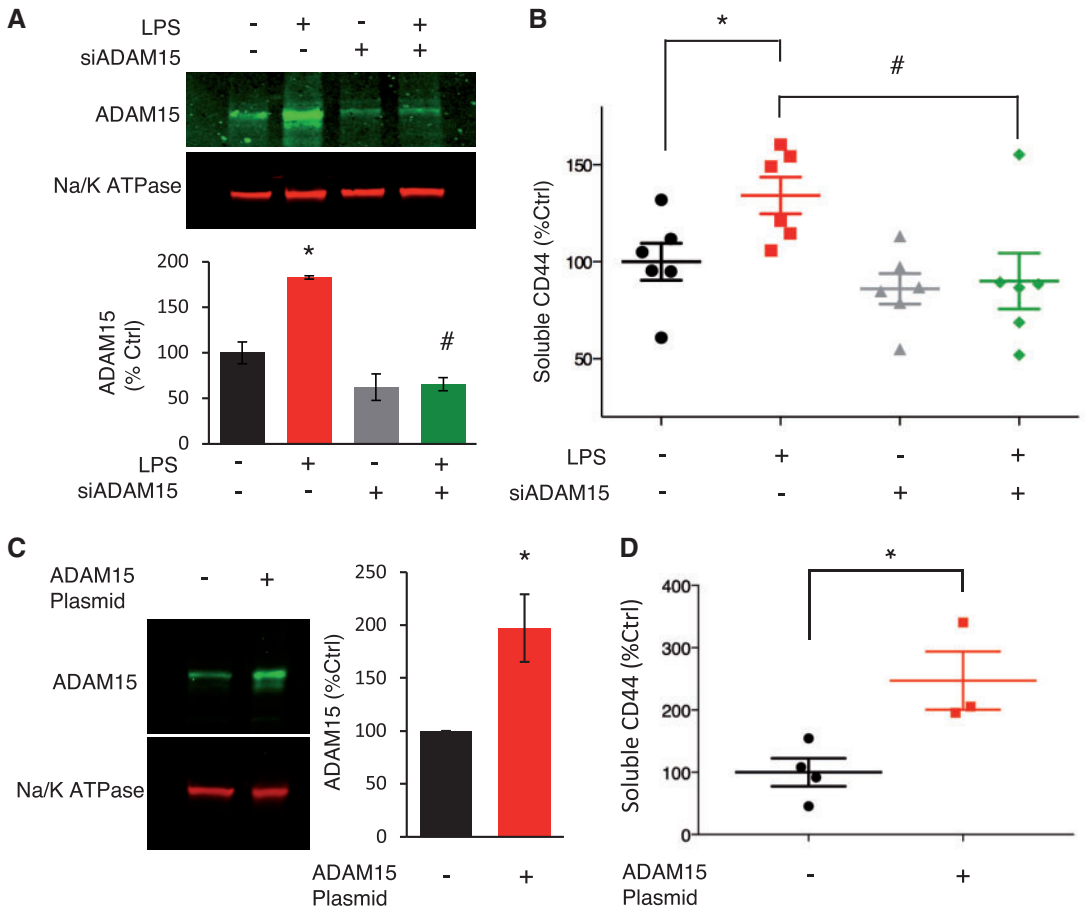

Figure 4 ADAM15 expression regulates CD44 ectodomain shedding. (*A*, *B*) The effects of ADAM15 knockdown on the shedding of CD44 ectodomain. (*A*) ADAM15 expression on cell membrane after LPS (1 μg/mL, 24 h) treatment in HUVECs with or without ADAM15 siRNA knockdown. Mean ± SEM, $n = 3$. Na/K ATPase serves as membrane fraction loading control. *$P < 0.05$ vs. vehicle + control siRNA, # $P < 0.05$ vs. LPS + control siRNA. (*C*, *D*) The effects of ADAM15 overexpression on CD44 ectodomain shedding. (*C*) Verification of ADAM15 overexpression (mean ± SEM, $n = 3$). Na/K ATPase = membrane fraction loading control. (*D*) The effects of ADAM15 overexpression on soluble CD44 level in HUVEC conditioned medium (mean ± SEM, $n = 3$). *$P < 0.05$ vs. control plasmid.

septic stimulation. These findings demonstrate that the endothelial glycocalyx plays an essential role in controlling vascular barrier function.

Destruction of the endothelial glycocalyx during sepsis has been documented in several studies. Serum concentrations of HA, syndecan-1, and heparan sulfate are significantly higher in patients with sepsis compared with healthy subjects.[25–27] Consistent with these findings, our data from *ex vivo* human lung perfusion experiments demonstrated that LPS perfusion resulted in a significant increase in the levels of soluble CD44 and HA in the perfusion effluent, indicating increased glycocalyx shedding in response to septic stimulation. Moreover, dextran exclusion assay showed that LPS treatment remarkably reduced the glycocalyx thickness in cremaster vessels. Schmidt *et al.*[14] applied the same technique to measure the glycocalyx thickness in pulmonary microvessels and reported a value about two-fold greater than our baseline. This discrepancy could be explained by the notable variation in glycocalyx dimensions among different organs. In fact, the glycocalyx thickness we measured is similar to the value measured in another study of cremaster vascular glycocalyx.[28]

To date, relatively little is known about the precise mechanism of glycocalyx degradation in various pathological conditions. Several enzymes have been implicated in this process, including heparanase, MMPs, hyaluronidase, etc.[29] Although many studies show that targeting these sheddases exerts beneficial effects in experimental models of sepsis, contradictory findings have been reported.[30,31] Unfortunately, there has been limited information on the clinical/therapeutic benefits of sheddase inhibitors in sepsis patients, as most of the clinical studies or trials have focused on the evaluation of these inhibitors in cancer patients, which have not reached a consensus. In this study, we found ADAM15 dose-dependently cleaved CD44, an important transmembrane glycoprotein that serves as an integral component of glycocalyx by linking HA and anchoring the glycocalyx layer to cell membrane. The ability of ADAM15 to cleave CD44 is further supported by data showing a positive correlation between the membrane expression of ADAM15 and the level of soluble CD44 in EC conditioned medium. *In vivo*, an elevated plasma level of soluble CD44 was detected in septic mice, consistent with our previous studies showing that sepsis induces

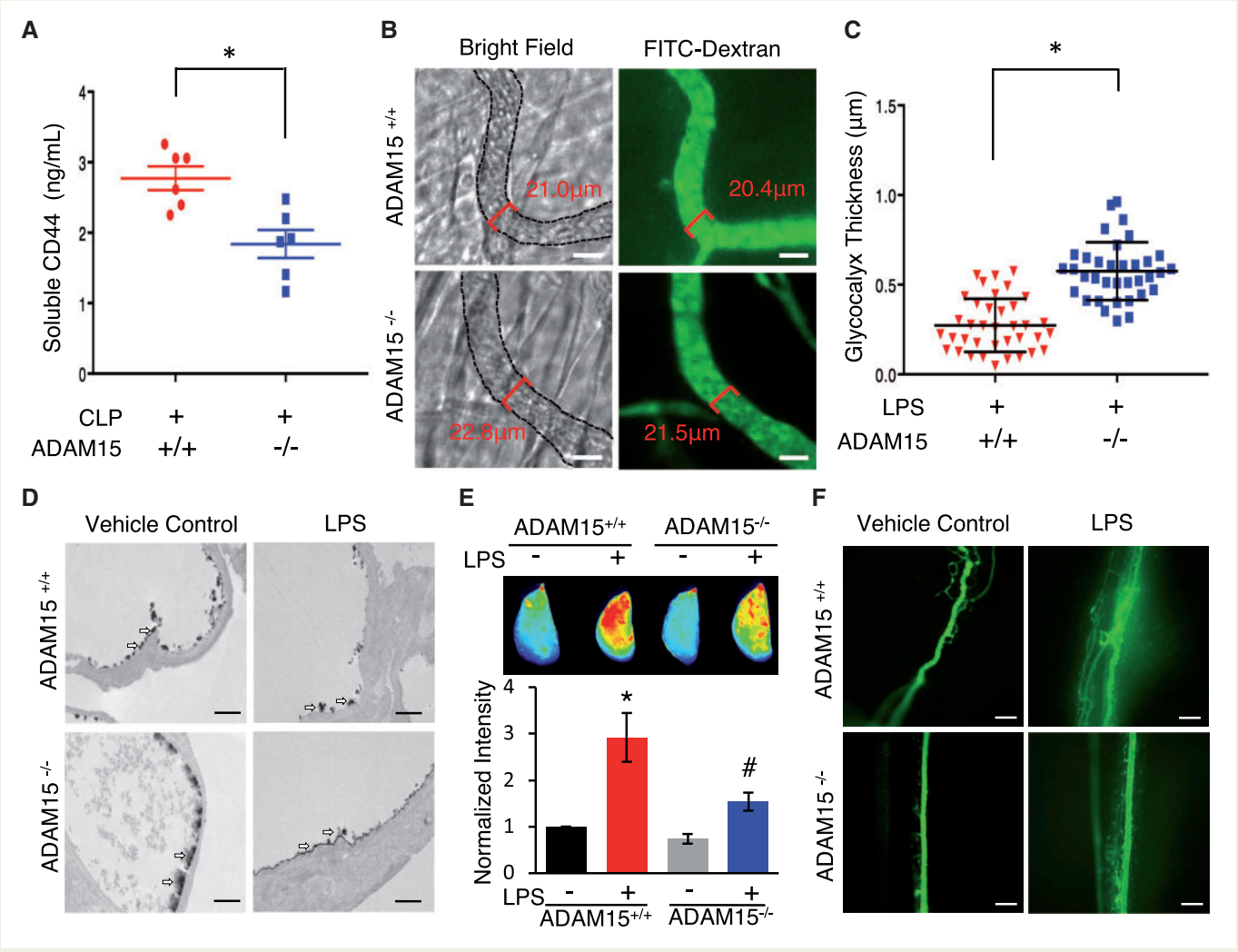

Figure 5 ADAM15$^{-/-}$ mice are resistance to sepsis-induced glycocalyx degradation and vascular leakage. (*A*) The plasma level of soluble CD44 in wild-type and ADAM15$^{-/-}$ mice after sepsis (mean ± SEM, $n = 6$). *$P < 0.05$. (*B*, *C*) The effects of ADAM15 deficiency on sepsis-induced glycocalyx shedding. (*B*) Representative images of mouse cremaster vessels in wild-type and ADAM15$^{-/-}$ mice subjected to LPS injection. Scale bar = 20 μm. (*C*) Quantification of the glycocalyx thickness (mean ± SD, $n = 5$ animals). Each dot represents the average of three measurements in one microvessel; *$P < 0.05$. (*D*) Electron microscopy images of pulmonary microvascular glycocalyx in wild-type and ADAM15$^{-/-}$ mice subjected to LPS challenge. Arrows indicated the endothelial glycocalyx. Scale bar = 1 μm. (*E*, *F*) The impacts of ADAM15 deficiency on LPS-induced vascular hyperpermeability. (*E*) Pulmonary vascular permeability was determined by Evans Blue extravasation assay 24 h after LPS injection. Evans Blue bound-albumin extravasation was quantified using LI-COR Odyssey Clx (mean ± SEM, $n = 4$). *$P < 0.05$ vs. vehicle + wild-type, #$P < 0.05$ vs. LPS + wild-type. (*F*) Intravital microscopy images showing FITC-albumin leakage from mesenteric microvessels ($n = 4$). Scale bar = 100 μm.

a significant up-regulation of ADAM15.[12] Kitti *et al.* and Komura *et al.*[32,33] have also reported an increased serum level of soluble CD44 in patients with inflammatory conditions, such as rheumatoid arthritis and systemic sclerosis. However, their studies have limited molecular insight into the soluble CD44 release. Our proteomic analyses identified the His^{235}-Thr^{236} bond in CD44 as a cleavage site of ADAM15, which is within the membrane-proximal region of CD44 extracellular domain. Upon cleavage by ADAM15, the ectodomain of CD44 is released into the circulation. Previous studies have mostly considered circulating soluble CD44 solely as a marker for diagnosis or a predictor for treatment responses. Little is known about the direct effects of soluble CD44 in the circulation. We demonstrate, for the first time, that the ectodomain of CD44 functions as an endothelial barrier-disruptive agent capable of inducing adherens junction disorganization. It is noteworthy that CD44 is a multifunctional receptor widely distributed in different cell types.[5] CD44 expressed on leukocytes has also been extensively studied and implicated as an adhesion molecule regulating neutrophil trafficking. In this study, we did not focus on testing the effect of ADAM15 on neutrophil CD44 with respect to its impact on neutrophil adhesion because our previous study showed minimal expression of ADAM15 in neutrophils.[34] However, as shown in the Supplementary material online, we did observe enhanced neutrophil migration across CD44 ectodomain-treated endothelial monolayers, which was likely attributable to junction opening, supporting our hypothesis about the signalling role of CD44 cleavage products in mediating endothelial paracellular hyperpermeability.

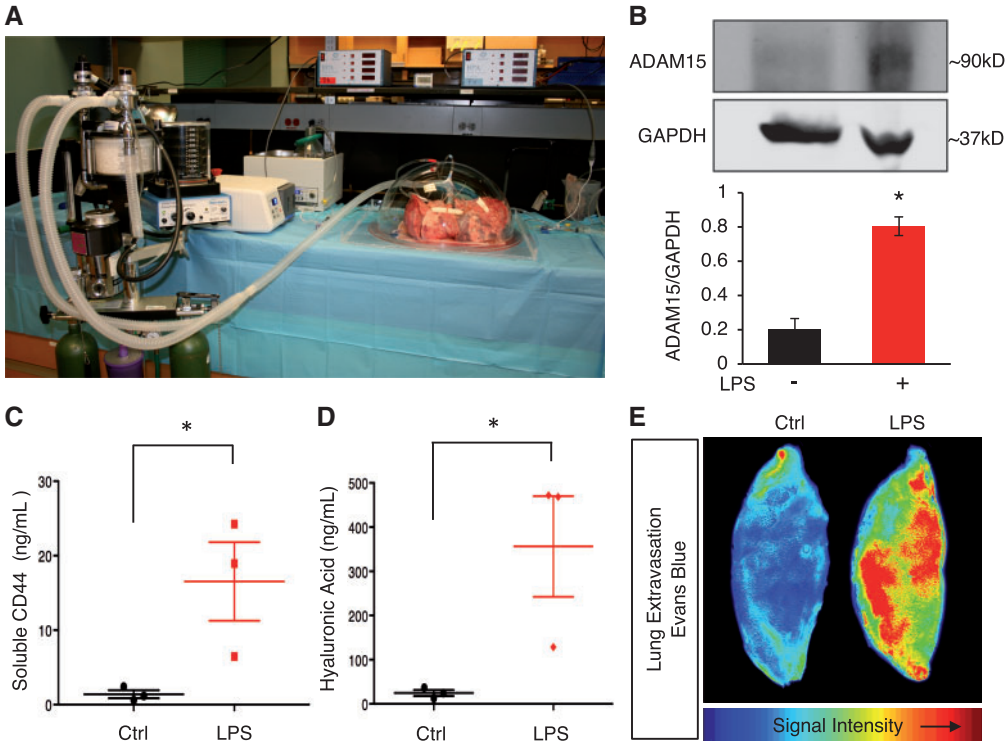

Figure 6 *Ex vivo* human lung subjected to LPS treatment exhibits increased glycocalyx damage and vascular hyperpermeability. (*A*) *Ex vivo* human lung perfusion system. Human lungs were ventilated and perfused with 0.25 mg/mL LPS for 6 h. (*B*) Western blot showing the expression of ADAM15 in human lung tissue with or without LPS treatment. GAPDH serves as loading control (mean ± SEM, $n = 3$). (*C*) The level of soluble CD44 level in the perfusion effluent (mean ± SEM, $n = 3$). (*D*) Circulating HA level in the perfusion effluent (mean ± SEM, $n = 3$). (*E*) Evans Blue extravasation assay showing human pulmonary microvascular permeability. $n = 3$. *$P < 0.05$.

Given the detrimental impact of glycocalyx damage to vascular function, more researchers turn their attention to protecting glycocalyx structure. Attempts to maintain the integrity of glycocalyx could be summarized into two categories: preventing degradation or promoting synthesis. The most extensively studied measure to prevent glycocalyx shedding is albumin administration. Despite the beneficial effects observed in several animal studies, [35] addition of albumin to crystalloids did not confer survival advantages in a controlled randomized-clinical trial with sepsis patients.[36] Another agent that may prevent glycocalyx injury is TNF-α inhibitor, Etancercept,[37] although its therapeutic effects remain to be determined. Sulodexide, a sulfated polysaccharide complex, has been shown to promote glycosaminoglycan synthesis and repair injured glycocalyx. However, in clinical trials with over 1000 diabetes patients, sulodexide failed to ameliorate albuminuria,[38] an indicator of renal glomerular hyperpermeability. This study suggests blocking ADAM15 as a potential option for maintaining glycocalyx integrity. This is supported by our data showing that the absence of ADAM15 attenuated sepsis-induced CD44 cleavage and the cremaster glycocalyx thinning. Furthermore, electron microscopy images provided direct evidence that lack of ADAM15 blocked LPS-induced reduction of glycocalyx coverage in mouse pulmonary vessels. More importantly, these protective effects of ADAM15 deficiency closely correspond to the attenuation of vascular hyperpermeability in the lungs and mesentery of septic mice.

There are limitations to this study. The first one lies in the technical difficulty in detecting the absolute concentration of cleaved CD44. We measured the level of soluble CD44 in the circulation or conditioned media as an indicator for CD44 cleavage. Although the majority of soluble CD44 in the plasma is composed of CD44 ectodomains as shedding products, we cannot fact out the possibility that a small portion of soluble CD44 represents its variants from alternative splicing. Given that soluble CD44 is well-accepted as a marker of CD44 cleavage in several fields, such as cancer metastasis, it is reasonable to consider soluble CD44 level an indicator of its cleavage under inflammatory conditions. Second, although not the focus of the current study, other sheddases or related molecular pathways may contribute to glycocalyx injury during sepsis. For instance, proteases including MMPs and ADAM17 have previously been implicated in the cleaving of glycocalyx components under inflammatory conditions[39,40] or other disease states.[9,41,42] Moreover, the reactive nitrogen species (RNS) derived from excessive nitric oxide (NO) produced during inflammation are capable of modifying both the core proteoglycans and glycosaminoglycan side chains of glycocalyx.[43] In addition to their direct effects on glycocalyx, NO and RNS may indirectly cause glycocalyx injury by activating MMPs.[44] The relative importance of the multiple glycocalyx degrading mechanisms in vascular barrier dysfunction and their detailed molecular/signalling mechanisms in septic injury remain to be determined in our future studies.

In summary, our results suggest that ADAM15 contributes to vascular endothelial barrier dysfunction by causing glycocalyx degradation.

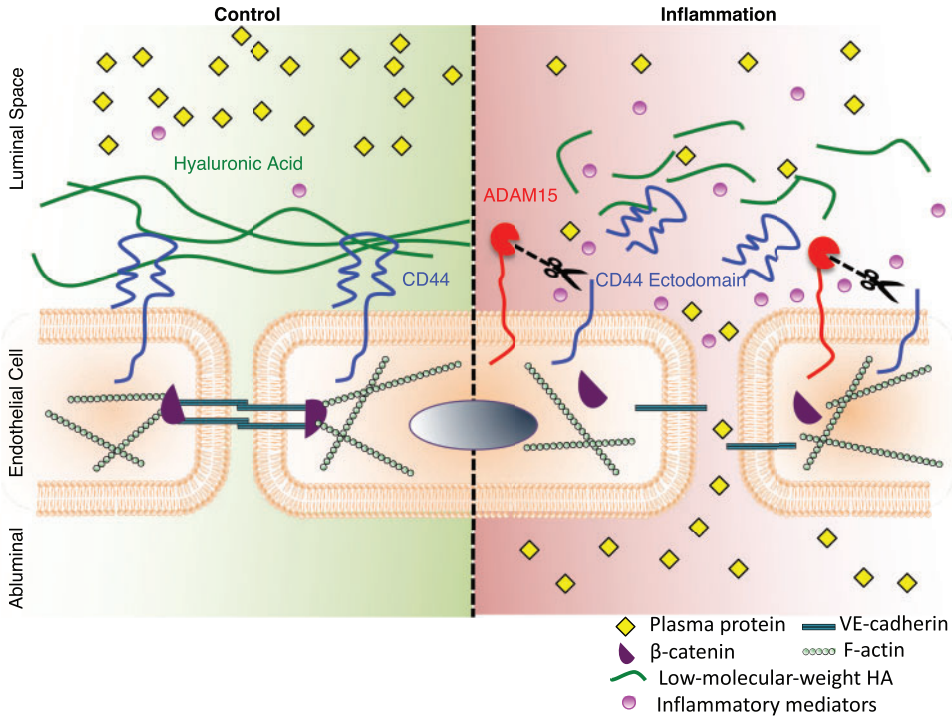

Figure 7 Schematic diagram showing ADAM15-induced glycocalyx damage contributes to vascular barrier disruption during inflammation. Under normal condition, the endothelial glycocalyx and the adherens junction at endothelial cell–cell contacts act collectively to prevent the transvascular leakage of plasma proteins. However, upon inflammation, up-regulated ADAM15 mediates the cleavage of CD44 via its metalloproteinase domain, resulting in the disruption of glycocalyx structural integrity. Reduced glycocalyx coverage impairs its barrier property against macromolecular leakage and increases the exposure of ECs to inflammatory mediators in the circulation. The cleaved products of glycocalyx (CD44 ectodomain and low-molecular-weight HA) cause adherens junction disorganization, which allows more plasma protein leakage into the interstitial space.

Its underlying mechanisms involve ADAM15-mediated CD44 cleavage and release of CD44 ectodomains into the circulation promoting vascular hyperpermeability.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

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