**Supplementary Materials and Methods**

*Cell culture – Human*

Human umbilical vascular endothelial cells (HUVECs) were purchased from Lonza (Basel, Switzerland) and grown in EGM-2 bulletkit media from Lonza (CC-3162) and VascuLife® VEGF Endothelial Medium Complete Kit from Lifeline technologies (LL-0003) (Frederick, MD) that contained 2% fetal bovine serum, 0.04% hydrocortisone, 0.4% hFGF-B, 0.1% VEGF, 0.1% R3-IGF-1, 0.1% ascorbic acid, 0.1% gentamicin amphotericin-b, and 0.1% heparin. Human lung microvascular endothelial cells (HLMVECs) were purchased from Lifeline technologies and grown in VascuLife® VEGF-Mv Endothelial media (LL-0005) with 5% fetal bovine serum that also contained 5 ng/mL rh FGF, 50 µg/mL ascorbic acid, 1 µg/mL hydrocortisone, 10 mM L-Glutamine, 15 ng/mL rh IGF-1, 5 ng/mL rh EGF, 5 ng/mL rh VEGF, 30 µg/mL gentamicin, 15 ng/mL amphotericin B, and 0.75 U heparin sulfate.

All cell types were grown in cell-culture treated multi-well plates from CytoOne (USA Scientific, Ocala, FL) at 5% CO2 and 37˚ C.

*Animal model - Mice*

Animal experimental studies were approved by the Institutional Animal Care and Use Committee at Rhode Island Hospital which followed the National Institutes of Health Guide for the Care and Use of Experimental Animals. All mice were purchased from Jackson laboratory (Bar Harbor, ME) and interbred in house. Wild type mice were on a C57BL/6J genetic background (Jackson #000664). *Cd44* knockout mice were of the strain *Cd44*tm1Hbg and are congenic on the C57BL/6J genetic background (Jackson #005085). *Prg4* gene trap knockout mice were of the strain *Prg4*tm2Mawa/J and have genetic contributions from both the C57BL/6J and 129S1/SvlmJ genetic backgrounds (Jackson #025740). Mice used for harvesting of MLMVECs were chosen from four different genotypes that were isolated from a total of 16 genotypes after interbreeding. The four genotypes that were interbred from the original 16 genotypes were WT (*Cd44*+/+/*Prg4*+/+), *Cd44*-/- (*Cd44*tm1Hbg*Prg4*+/+), *Prg4*GT/GT (*Cd44*+/+ *Prg4*tm2Mawa/J), and double knockout (DKO: *Cd44*tm1Hbg *Prg4*tm2Mawa/J). Mice were raised on a 12:12 light cycle and were fed a standard rodent diet.

*Cell Culture – mouse*

Mouse lung microvascular endothelial cells (MLMVECs) were harvested via magnetic bead separation from four different genotypes of mice including wild type (WT: *Cd44*+/+*Prg4*+/+ ), *Cd44* -/-( *Cd44*tm1Hbg*Prg4*+/+), *Prg4* GT/GT (*Cd44*+/+ *Prg4*tm2Mawa/J), and double knockout for both *Cd44* and *Prg4* (DKO: *Cd44*tm1Hbg *Prg4*tm2Mawa/J). MLMVECs were grown in Lifeline technologies VascuLife® VEGF-Mv Endothelial media as described under cell culture.

*MLMVEC harvest*

*Sort 1*

The MLMVECs were harvested with a modified protocol based upon similar procedures [1, 2]. Transgenic mice were used between the ages of 3-10 weeks for lung collection. Five to 6 mice of both sexes from each genotype were euthanized with CO2 and lungs were removed via excision and placed into isolation media #1 (high glucose DMEM + 1x penicillin/streptomycin solution) on ice. The following procedures, including sort 2, were performed in a laminar flow hood. Lungs were very finely minced and incubated for 45 minutes in Type 1 collagenase (CSL-1 Worthington Biochemical Corporation, Lakewood, NJ, USA) at 37˚C. The tissue was then triturated with a 16G cannula 15 times before passing through a 70 µM cell strainer. The cell strainer was rinsed with 15 mL isolation medium 2 (high glucose DMEM + 1x penicillin/streptomycin + 20% FBS). Cells were pelleted via centrifugation at 400g x 5 minutes and then washed with bead wash (0.1% BSA in PBS with 1x penicillin/streptomycin) twice before incubating cells with anti-PECAM-1 (CD31) antibody (#553369, BD Biosciences, San Jose, CA, USA) conjugated Dynabeads (ThermoFisher Scientific, USA). The mixture was tumbled at RT for 15 minutes before the cells were rinsed with bead wash 4x on the DynaMag™(ThermoFisher Scientific, USA). The cells were then seeded onto a CytoOne T-75 flask that was pre-coated with rat tail collagen (Cell Applications, San Diego, CA, USA) and media was changed the next day and every other day following. When cells were at least 80% confluent, they were detached from the flask using 0.05% trypsin and used for the following procedure.

*Sort 2*

Trypsinized cells were centrifuged at 400g x 5 minutes and rinsed with bead wash. Cells were incubated with anti-ICAM2 (CD102) (#553325, BD Biosciences, San Jose, CA, USA) Dynabeads mix and tumbled at RT for 15 minutes. Cells were washed on the DynaMag™ 4x with bead wash then seeded onto a T-75 flask that was coated with rat tail collagen and onto a chamber slide coated with fibronectin (Corning™ BioCoat™ Fibronectin Culture Slide, NY, USA). Media was changed the following day and every other day thereafter. Cells from the culture flask were used for ELISA and the cells on the culture slide were used for immunofluorescence.

*Immunofluorescence*

Cells were grown to 80% confluency on chamber slides. Media was removed and 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) was added to each chamber and incubated for 20 minutes at RT. The cells were washed twice with PBS and twice with wash buffer (0.1% BSA (Sigma-Aldrich) in PBS). Non-specific staining was reduced by incubating the cells in blocking buffer for 1 hr at RT in 10% normal donkey serum (Sigma-Aldrich) and 0.3% Triton X (Sigma-Aldrich) in PBS. Blocking buffer was removed and cells were incubated in 1:250 rabbit anti-mouse VE-Cadherin (PA5-19612, Invitrogen, USA) in dilution buffer (1% BSA and 0.3% Triton X-100 in PBS) for 1 hr at RT. Cells were washed 2x with wash buffer and incubated in 1:500 donkey anti-rabbit IgG Alexa Fluor™ Plus 555 (A32794, Invitrogen, USA) for 1 hr at RT. Cells were washed 2x in wash buffer, once with PBS and once with dH2O. The gasket was removed and slide was cover-slipped with VECTASHIELD® Antifade Mounting Media with DAPI (Vector labs, Burlingame, CA, USA). Negative controls were conducted that were incubated with the secondary antibody but not the primary antibody.

*Imaging*

Cells immunolabeled for VE-Cadherin were imaged using a Nikon E800 microscope with 20x objective lens and a Spot RT3 camera from Diagnostic Instruments (Sterling Heights, MI, USA). Ten fields were chosen from each genotype of mouse cell and cell counts were compared to total cell number positive for VE-cadherin using ImageJ.

*LPS and rhPRG4 exposure*

When cells were approximately 80% confluent, they were exposed to either 100 or 250 ng/mL Ultrapure LPS from E. coli K12 (Invivogen, San Diego, CA, USA) for 30 minutes prior to cell treatment with recombinant human proteoglycan 4 (rhPRG4) for 23.5 hours. rhPRG4 is endotoxin free and produced by CHO-M cells [3] (Lubris, Framingham, MA, USA). Media was collected for ELISA and in some cases, cells were collected for RNA extraction to perform qPCR for ΔΔCt analysis.

*Patient samples*

Patient plasma samples were obtained from SEA biobank at Rhode Island hospital under IRB protocol # 4116-16 and used to treat both HUVEC and HLMVEC. SEA 4, 11, 15, 22, 23, 30, 34, and 41 were all culture positive for *E. coli*. SEA culture 30 was also positive for Coagulase-negative *staphylococci*. SEA 5, 8, 12, 13, 14, 20 and 24 were culture negative samples. SEA denotes “Sepsis, ECMO, and ARDS”.

*IL-6 ELISA*

IL-6 protein levels in the cell culture media were assessed using Abcam (Cambridge, MA, USA) SimpleStep Human IL-6 ELISA Kit (ab178013: kit detection limit 7.8-500 pg/mL) and mouse IL-6 ELISA kit (ab222503: kit detection limit 15.6-1000 pg/mL) and were used per manufacturer’s instructions. Cell culture media was centrifuged at 2000g x 10 mins and supernatant was analyzed by ELISA for IL-6 protein. The media was diluted in the kit sample diluent and triplicates were run of each sample and compiled from two independent experiments. Following the protocol completion, the optical density was measured at 450 nm on a SpectraMax 190 (Molecular Devices, San Jose, CA). Data are reported as mean ± SEM.

*PRG4 ELISA*

PRG4 levels in control and sepsis patient plasma were measured via an inhibition ELISA, which is described by Minrong et al. [4]. Briefly, 100µl of 10 µg/ml of rhPRG4 in PBS was added and incubated overnight at 4°C in 96 well plates. Plates were washed 3 times with 200 µl PBST. Blocking was performed with 200 µl of 5% BSA in PBST for 2 hours at RT with shaking and plates were washed as described above. An rhPRG4 stock solution of 100 µg/ml was created with serial dilutions to make a standard curve. 50 µl of diluted standards or samples and 50 µl of 1:3000 anti-PRG4 antibody 9G3 (Millipore, Burlington, MA, USA) in PBS were added to each well and incubated for 1 hr at RT with shaking and washed as above. Goat anti-mouse IgG (Life Technologies, Carlsbad, CA, USA) was diluted to 1:3000 in PBS and 100µl was added to each well and incubated for 1 hr at RT and washed 5x as above. 100µl of TMB solution (ThermoFisher Scientific, Waltham, MA, USA) was added to each well and allowed to activate for 10 min. Then 100µl of 1M H3PO4 was added to each well to stop the reaction and plates were read at 450nm using a SpectraMax 190. Triplicates were collected for each standard and sample and were compiled from three independent experiments. Data were compiled into two groups consisting of control patients and sepsis patients and a one-tailed Welch’s t-test was performed. Data are reported on a semi-log graph as nested data points overlaying the mean ± SEM.

*IL-6 Gene expression*

Following treatment with patient plasma and rhPRG4, cells were rinsed with PBS. Cells were treated with 0.05% trypsin until cells detached from the cell culture plate. Media was added and then cells were collected and placed in 15mL tubes and centrifuged for 5 minutes at 300g. RNA was then collected from cells in culture using the Qiagen (Germantown, MD, USA) RNeasy mini kit and stored at -80 ˚ C until reverse transcription (RT). The RT was performed using the Qiagen QuantiTect Reverse Transcription Kit. RNA concentrations were measured using the NanoDrop ND-2000c spectrophotometer (NanoDrop Technologies, USA). cDNA was synthesized using the Qiagen QuantiNova Reverse Transcription Kit. qPCR was performed using Qiagen QuantiTect Primer Assays IL-6 (NM\_000600, XM\_005249745) and RRN18S (X03205) along with the Qiagen QuantiNova SYBR Green PCR Kit. The Eppendorf (Hamburg, Germany) Realplex4 mastercycler was used to determine Ct in order to calculate 2-ΔΔCt[5]. Cycling conditions were 95 ˚ C for 2 minutes and then 40 cycles of 95 ˚ C for 5 seconds and 60 ˚ C for 10 seconds. Melting curve analysis was performed following qPCR. Data are reported as fold target gene expression compared to the untreated control group. Gene expression was assayed in triplicate.

*Statistical analysis*

Prism© (GraphPad, San Diego, CA) was used for statistical analysis. A p value of <0.05 was considered statistically significant. ELISA data was compared via unpaired t-tests for comparisons within groups or ANOVA for comparisons between groups followed by post-hoc multiple comparisons using the Dunnett test unless otherwise noted. Gene expression analysis results were considered significant if the ΔΔCt values were below 0.5 or above 2. A Pearson’s correlation was computed to estimate the relation between PRG4 and IL-6.

References

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