SUPPLEMENTARY MATERIAL

REAGENTS

Antibodies for immunofluorescence

The following primary antibodies were used for IF: sheep anti-human VWF (1:1,000 dilution, Abd Serotec, Oxford, UK, AHP062), rabbit anti-human VWF (1:300 dilution, Dako, Carpinteria, CA, A0082), goat anti-human P-selectin (CD62P, 1:100 dilution; Santa Cruz Biotechnology, Dallas, TX; sc-6943), PE conjugated mouse anti-human CD144 (VE-Cadherin, 1:200 dilution, BD Pharmingen, San Diego, CA), rabbit polyclonal antibody to MCP (1:50 dilution; Santa Cruz Biotechnology, Dallas, USA; sc-9098), rat polyclonal antibody to CD59 (1:1,000 dilution; Abd Serotec, Oxford, UK; MCA715G), and goat polyclonal antibody to CD55 (1:50 dilution; R&D Systems, Minneapolis, MN; AF2009). Nuclei of cells were stained with 0.12 μg/ml Hoechst stain (Thermo Fisher Scientific, Waltham, MA) for 10 minutes.

Antibodies for flow cytometry

The following primary antibodies were used for BOEC characterization: Alexa Fluor 488 conjugated mouse anti-human CD31 (PECAM-1) [5 μL/test], PE conjugated mouse anti-human CD144 (VE-Cadherin) [12 μL/test], Alexa Fluor 647 conjugated mouse anti-human CD14 [5 μL/test] and APC-H7 conjugated mouse anti-human CD45 [3 μL/test] were used for surface staining of the BOEC, with Alexa Fluor 488 mouse IgG2 κ, PE mouse IgG1 κ, Alexa Fluor 647 mouse IgG2b κ and APC H7 mouse IgG1 κ, as isotype controls. Antibodies were purchased from BD Pharmingen (San Diego, CA).

For detection of complement regulators we used rabbit anti-CD46, 1:50 dilution, Santa Cruz Biotechnology, Dallas, TX, sc-9098; goat anti-CD55, 1:100 dilution, R&D Systems, Minneapolis, USA, AF2009 and rat anti-CD59, 1:100 dilution, AbD Serotec, Oxford, UK, MCA715G.

For detection of complement deposition we used a rabbit polyclonal anti C3c- antibody directly conjugated with FITC from abcam (ab4212). For sensitization with β2 microglobulin we used a polyclonal goat anti-C3 antibody (A213) from Complement Technology, Tyler, TX.

Primers for RT-qPCR

The following oligonucleotide primers (Sigma-Aldrich, Carlsbad, USA) were used:

GAPDH: forward, 5'-ACAGTTGCCATGTAGACC-3'; reverse, 5'-TTTTTGGTTGAGCACAGG-3'

VWF: forward, 5'-TGTATCTAGAAACTGAGGCTG-3'; reverse, 5'-CCTTCTTGGGTCATAAAGTC-3'

CD46: forward, 5'-AGTGGTCAAATGTCGATTTC-3'; reverse, 5'-ATCCCAAGTACTGTTACTGTC-3'

CD55: forward, 5'-CAGAGGAAAATCTCTAACTTCC-3'; reverse, 5'-AGTTGGTGAGACTTCTGTAG-3'

CD59: forward, 5'-CATTACCAAAGCTGGGTTAC-3'; reverse, 5'-TTTCTCTGATAAGGATGTCCC-3'

VWF: forward, 5'-TGTATCTAGAAACTGAGGCTG-3'; reverse, 5'-CCTTCTTGGGTCATAAAGTC-3'

METHODS

Immunofluorescence

Cells were seeded onto collagen-coated 22 x 22-mm cover slips (VWR International, Radnor, PA) at a concentration of 0.5×10^6 cells/ml and incubated overnight, washed with ice-cold PBS followed by fixation with 4% (w/v) paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) in PBS and blocked with 1% (w/v) BSA/PBS either alone or with 0.2% (v/v) Triton X-100 (for permeabilization) for 60 minutes. Samples were stained overnight with the primary antibodies made up in the 1% (w/v) BSA/PBS solution, followed by incubation for 1 hour with respective species specific donkey-anti secondary antibodies conjugated with Alexa Fluor® 488 or Alexa Fluor 555 (1:1,000 dilution, Invitrogen, Life Technologies, Carlsbad, CA). Cell nuclei were stained using 0.12 µg/ml Hoechst 33342 stain (Thermo Fisher Scientific, Waltham, MA) for 10 minutes. Cover slips were mounted with Dako Fluorescence Mounting Media (Dako Canada, Burlington, Ontario, Canada) for microscopy. Images were taken with a spinning disk confocal microscopy equipped with an Olympus IX81 inverted fluorescence microscope using a 60./1.35 oil immersion objective equipped with a Hamamatsu C9100-13 back-thinned EM-CCD camera and Yokogawa CSU X1 spinning disk confocal scan head (with upgrade from Spectral Aurora Borealis, Richmond Hill, Ontario, Canada). The unit is equipped with 4 separate diode-pumped solid state laser lines (Spectral Applied Research, Richmond Hill, Ontario, Canada, 405 nm, 491 nm, 561 nm, and 642 nm) with emission filters: 447 nm ± 60, 525 nm ± 50, 593 nm ± 40, 620 nm ± 60, 676 ± 29 and 700 nm ± 75, and 1.5x magnification lens (Spectral Applied Research). Confocal images were taken with an Improvision Piezo Focus Drive. Z-stacks were taken at 0.25 µm. Images were then deconvolved by iterative restoration

using Volocity Software (PerkinElmer, Waltham, MA) with confidence limit set to 95% and iteration limit set to 20.

Flow cytometry for complement regulators and C3c deposition

Cells were seeded overnight in a 6 well plate to confluence, washed 1x with HBSS and then trypsinized for 1 minute (0.05% Trypsin/0.53 mM EDTA, WISENT, St Bruno, Canada). Primary antibodies and secondary antibodies (Alexa fluor 488, 1:200 dilution for complement regulators, Invitrogen, and R-Phycoerythrin-conjugated AffiniPure F (ab')2 Fragment Donkey Anti-Rabbit IgG (H+L) 709-116-149 [Jackson ImmunoResearch Laboratories, West Grove, PA] 1:200 for complement deposition experiment) were incubated at 4°C for 20 minutes. At least 10,000 events of BOEC population were recorded using Attune Acoustic Focusing Cytometer (Life Technologies) and analyzed using FlowJo software. Results are given as median fluorescence intensity (MFI). Cells were gated for live cells (red laser 536 nm, emission channel 2), single cells (FSC-A vs. FSC-H) and finally through forward scatter and side scatter to determine the BOEC population. C3c was recorded via the blue laser 488 nm, emission channel 2. To correct for spectral overlap during multicolor flow cytometry experiments, color compensation was performed each time.

Flow cytometry for endothelial cell phenotype

Cells were stained with primary antibodies and Fixable Viability Dye eFluor 450 (eBioscience, San Diego, CA), fixed with 2% PFA/PBS and blocked with 200 µg/mL final volume of purified mouse serum IgG (Sigma-Aldrich, St. Louis, MO) prior to specific or isotype antibody incubations. A minimum of 60,000 live cells was collected using a Beckman Coulter Gallios

flow cytometer (Beckman Coulter, Brea, USA), equipped with 4 excitation lasers (405, 488, 561 and 633 nm). Data was analyzed with Kaluza software (Beckman Coulter). HUVECs passage 6 were used as a positive control for the endothelial cell markers CD31 (PECAM-1) and CD144 (VE-Cadherin) and blood as a positive control for CD45 and CD14. Fluorescence minus one (FMO) controls using BOEC were stained for each of the four specific antibodies and were used as the gating controls during data analysis.

Quantifying Gene Expression by RT-qPCR

Cells (BOEC, HUVEC and GEC) were seeded overnight in a 6 well plate to confluence, washed 1x with HBSS before RNA was isolated using TRI Reagent (Sigma-Aldrich, St. Louis, MO, T9424) according to manufacturer's instructions. RNA concentration and integrity was verified by spectrophotometer (NanoDrop 1,000, Thermo Fisher Scientific, Waltham, MA), and reverse transcribed using ReadyScript™ cDNA Synthesis Mix (Sigma-Aldrich). Samples (200 ng cDNA in diethyl pyrocarbonate [DEPC, Sigma-Aldrich] treated water) were amplified by real time polymerase chain reaction (RT-PCR) using StepOne™ System from Life Technologies (Carlsbad, USA). Amplified products were detected using KiCqStart™ SYBR® Green qPCR ReadyMix™, with ROX™ (Sigma-Aldrich) and analyzed as follows: 2 – (CT – CT GAPDH) – CT control).

Normal human serum

50% normal human serum (50% NHS) was used as source of complement. Serum was collected from whole blood of adult donors into serum vacutainers (BD Biosciences), allowed to clot for 30 minutes and then centrifuged at 3,000 x g at 4°C for 10 minutes. Serum was stored at -20°C

until needed. Heat-inactivated serum (HIS), for use as a negative control, was obtained by incubating the serum for 30 minutes at 56°C.

Platelet isolation and perfusion

In brief, whole blood was mixed in a 6:1 ratio with acid citrate dextrose (ACD: 22.9 mM citric acid, 44.9 mM sodium citrate dehydrate, 74 mM dextrose monohydrate). Platelet rich plasma (PRP) was collected after centrifugation at 160 x g for ten minutes. PRP was spun at 950 x g for seven minutes to pellet platelets. Platelets were washed twice with PBS/ACD solution (20% ACD in PBS; pH 6.1). Platelets were incubated with 2.5 μM calcein (Life Technologies, Carlsbad, CA) for 30 minutes at 37°C. Finally, platelets were pelleted at 950 x g and resuspended at a concentration of 15 x 10^7/ml in Tyrodes buffer (136 mM NaCl, 2.7 mM KCl, 0.42 mM NaH2PO4, 19 mM NaHCO3, 5.5 mM of glucose, 1 mM CaCl2, 1 mM MgCl2 and 10 mM of hyroxyethyl piperazineethanesulfonic acid [HEPES: Invitrogen, Life Technologies, Carlsbad, CA]). Platelet count was measured using an automatic hematocytometer (Beckman Coulter, Brea, CA).

Briefly, BOECs were seeded into the BioFlux System (Fluxion Biosciences, South San Francisco, CA) channels the day before the actual experiment. BOECs were grown in BioFlux 48 well tissue culture plates coated with 0.05 mg/ml rat-tail collagen type I (Becton Dickinson). The collagen was added to the output well and flown backward at a shear rate of 3 dyne/cm^2 for 1-2 minutes after which the plate was incubated overnight at 37°C. BOECs were trypsinized and concentrated to 8 x10^6 cells/mL. After adding 50 μl of cEGM-2 to the input well as a balance, 50 μl of the cell solution were transferred to the output well and then perfused backwards for 15 seconds at 1 dyne/cm^2. After sufficient cell adhesion was observed under the microscope, the

plate was put into the 37°C incubator for 1 hour before adding 1 ml of cEGM-2 to each input well. Cells were left in a 37°C / 5% CO_2 incubator overnight.

BOEC lysates

Fully confluent BOECs and GECs were lysed using RIPA buffer with 2x protease inhibitor cocktail (Roche).

FIGURE LEGENDS (see Proofs)