PNAS
www.pnas.org

Supplementary Information for

Podocalyxin is required for maintaining blood-brain barrier function during acute inflammation

Jessica Cait, Michael R. Hughes, Matthew R. Zeglinski, Allen W. Chan, Sabrina Osterhof, R. Wilder Scott, Diana Canals Hernaez, Alissa Cait, A. Wayne Vogl, Pascal Bernatchez, T. Michael Underhill, David J. Granville, Timothy H. Murphy, Calvin D. Roskelley and Kelly M. McNagny

Kelly M. McNagny and Michael R. Hughes
Email: kelly@brc.ubc.ca, mhughes@brc.ubc.ca

This PDF file includes:

Methods for SI
Figs. S1 to S8
Captions for movies S1 to S8

Other supplementary materials for this manuscript include the following:

Movies S1 to S8

www.pnas.org/cgi/doi/10.1073/pnas.1814766116

Supplementary Methods

Apoptosis assay. CTRL and PODXL^{KD} HUVEC were plated on 12-well tissue culture-treated plastic plates coated with ECM components (fibronectin 10 µg/mL, laminin 5 µg/mL, collagen 1% gelatin) as indicated in the figures. On day of harvest, cells were lifted with 0.025% trypsin, washed twice with PBS and stained with Annexin V and propidium iodide (PI) to assess apoptosis (BD Pharmingen, Cat#556547). Staining was analysed using flow cytometry.

Poly(I:C) administration. Polyinosinic-polycytidylic acid (poly(I:C)) (Sigma, P1530) prepared in PBS (2 mg/mL) was administered to mice (10 mg/kg, i.p. route). After 6 h Poly(I:C) (or PBS) treated mice were treated with 5 mg/kg PAR-1 agonist (TFLLR-NH_2, SCP0237) (Sigma) prepared in PBS or PBS alone (i.v., tail vein). Mice treated with the PAR-1 agonist were observed for changes in behaviour and video recorded for subsequent analysis. Mice treated with PBS did not demonstrate any change in activity. Activity was scored by reviewing videos based on total motion observed at 10 s intervals (with 1=normal, 0.5=reduced activity and 0=no activity) and time for fully recovery (sustained return to score = 1).

Immunohistochemical staining of podocalyxin. Naïve mice were anesthetized with 2,2,2-tribromoethanol and then mice were perfused with PBS followed by 10% formalin (10 mL each) through the cardiac right ventricle. Skulls were removed and post-fixed in 4% PFA at 4°C for 24 h. The brains were removed, transferred to 70% ethanol and paraffin embedded. Tissue sections (5-10 µm) were cut and mounted. Sections were then deparaffinised and rehydrated. 3,3′-Diaminobenzidine (**DAB**) immunohistochemical staining was performed using an anti-podocalyxin antibody (R&D, AF1556) and anti-goat biotinylated secondary antibody (Vector labs, BA-500). Imaging was performed using a Nikon epifluorescence microscope (Nikon eclipse Ni-U).

Blood pressure and heart rate monitoring. Tail-cuff blood pressure and heart rate was monitored using CODA non-invasive blood pressure system (Kent Scientific, Torrington, CT). LPS-treated mice were placed in restrainers on a heating pad and baseline measurements were established over 10-20 measurement cycles. Mice were then treated with 5 mg/kg PAR-1 agonist (TFLLR-NH_2, SCP0237) (Sigma) prepared in PBS or PBS alone (i.v., tail vein). Mice were quickly placed back on the blood pressure system and monitored again for a 5 min period.

Calculation of EEG power suppression. The time course of mean spontaneous EEG power (0.5-4 Hz) was calculated for each experiment offline using Matlab software (Mathworks, Natick, MA). Maximal inhibition was determined as the mean EEG power minima occurring within 10 min following PAR-1 agonist injection. Only minima that exceeded a threshold (baseline mean EEG power minus two-times the standard deviation) were considered. Minima that did not exceed the threshold were assigned baseline values.

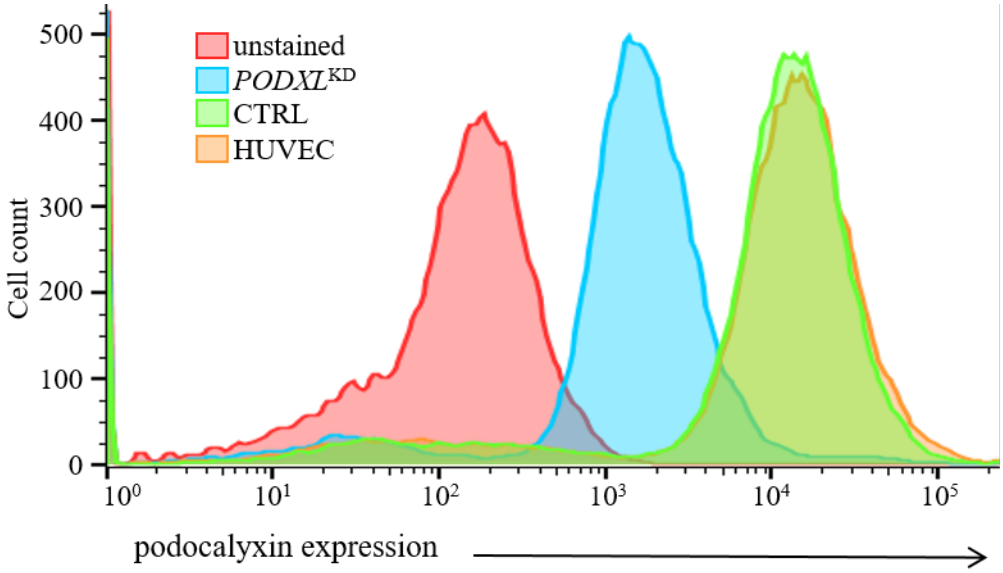


Fig. S1. siRNA knockdown of podocalyxin in HUVEC. Knockdown assessed by flow cytometry. PODXL^{KD} cells showed a 90% knockdown of podocalyxin surface expression. Percentage knockdown was calculated using mean florescence intensity (MFI) of the geometric mean. Representative calculation was done as follows: Unstained MFI=66.7, Podxl^{KD} MFI= 832, CTRL= 5141. 100 - ((CTRL-US)/(Podxl^{KD}-US)) = 93.4% knockdown.

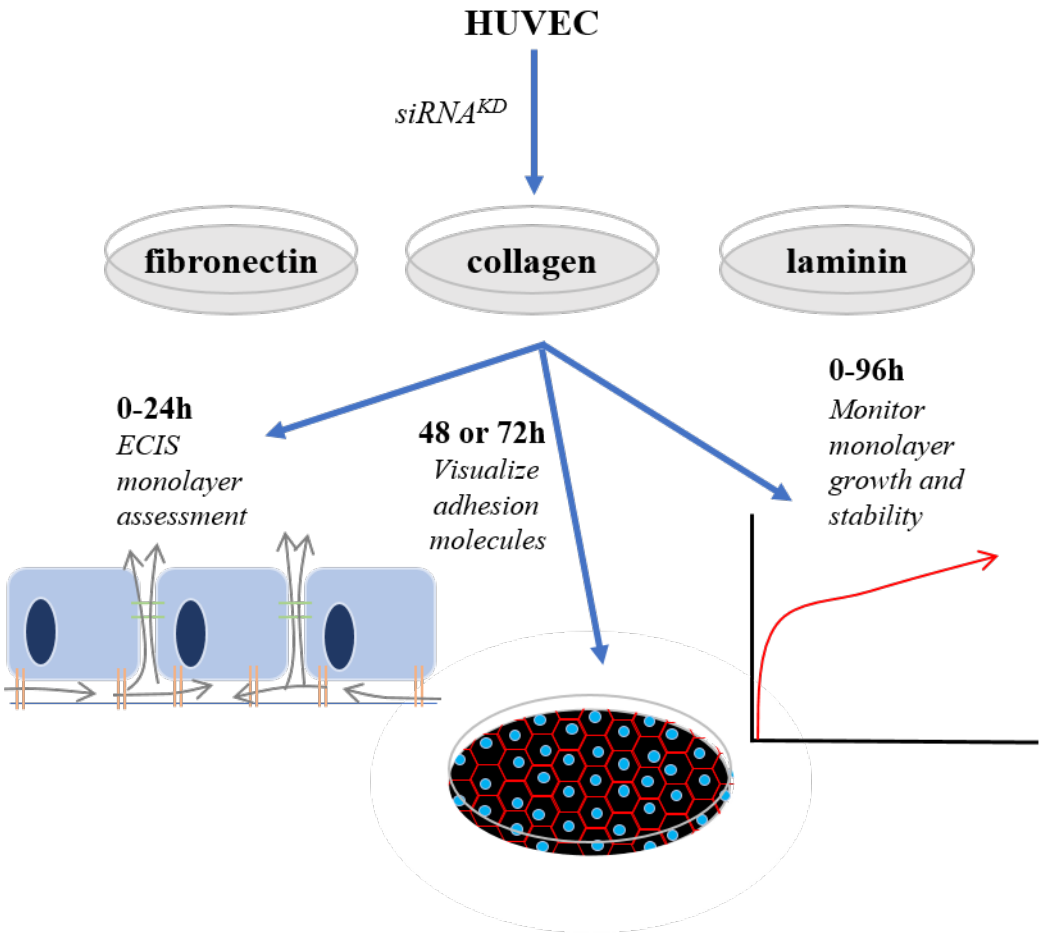
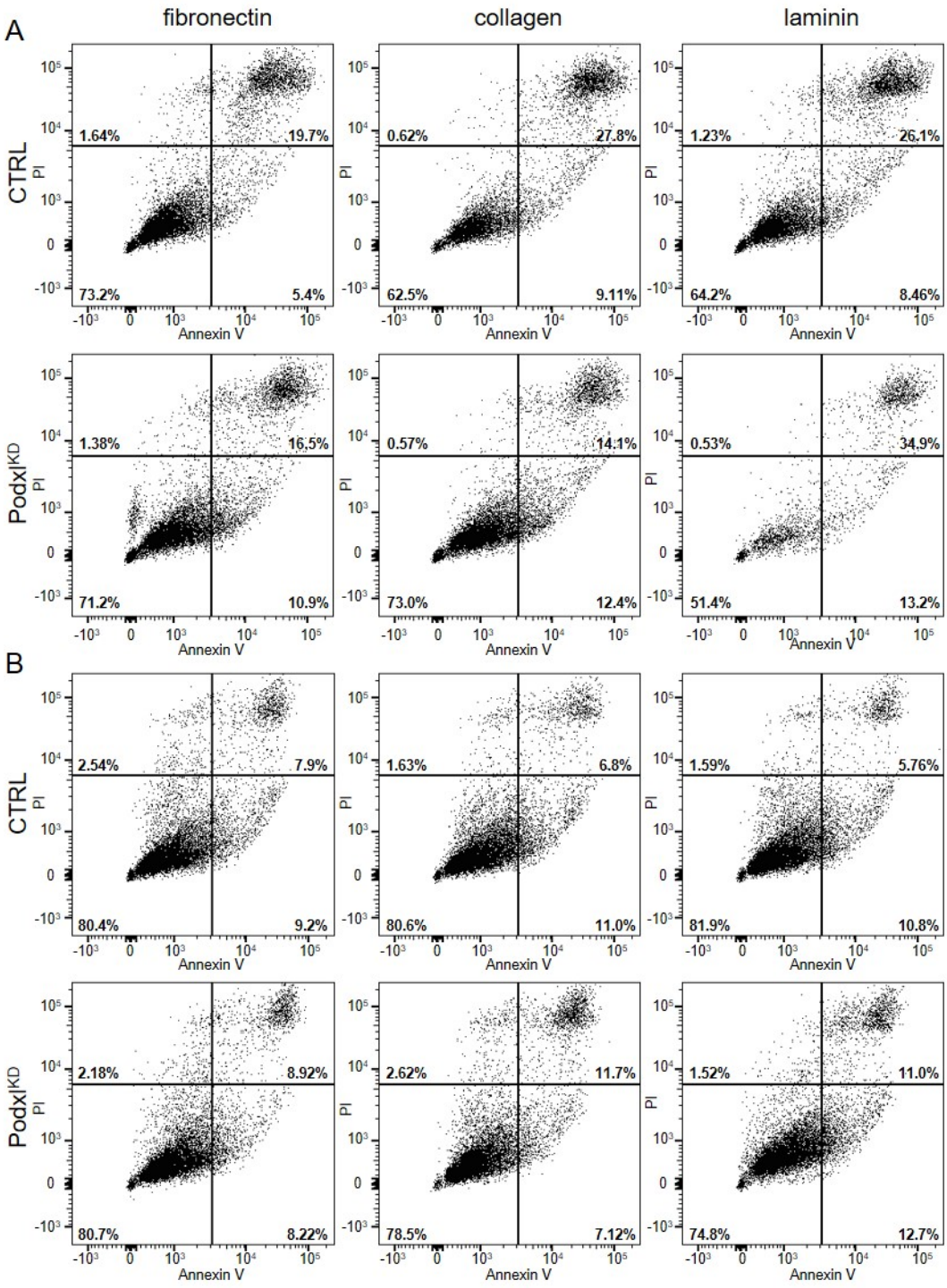


Fig. S2. Schematic representation of *in vitro* experiments. Podocalyxin expression was suppressed using siRNA transfection in HUVEC. Cells were subsequently plated on individual matrix components for the designated times for assay of electrical barrier function (0-24 h), localization of adhesion molecules (48 or 72 h) or maintenance of monolayers (0-96 h).


A
fibronectin
collagen
laminin
CTRL
Podx^KD
PI
Annexin V
1.64%
19.7%
73.2%
5.4%
0.62%
27.8%
62.5%
9.11%
1.23%
26.1%
64.2%
8.46%
1.38%
16.5%
71.2%
10.9%
0.57%
14.1%
73.0%
12.4%
0.53%
34.9%
51.4%
13.2%
B
2.54%
7.9%
80.4%
9.2%
1.63%
6.8%
80.6%
11.0%
1.59%
5.76%
81.9%
10.8%
2.18%
8.92%
80.7%
8.22%
2.62%
11.7%
78.5%
7.12%
1.52%
11.0%
74.8%
12.7%

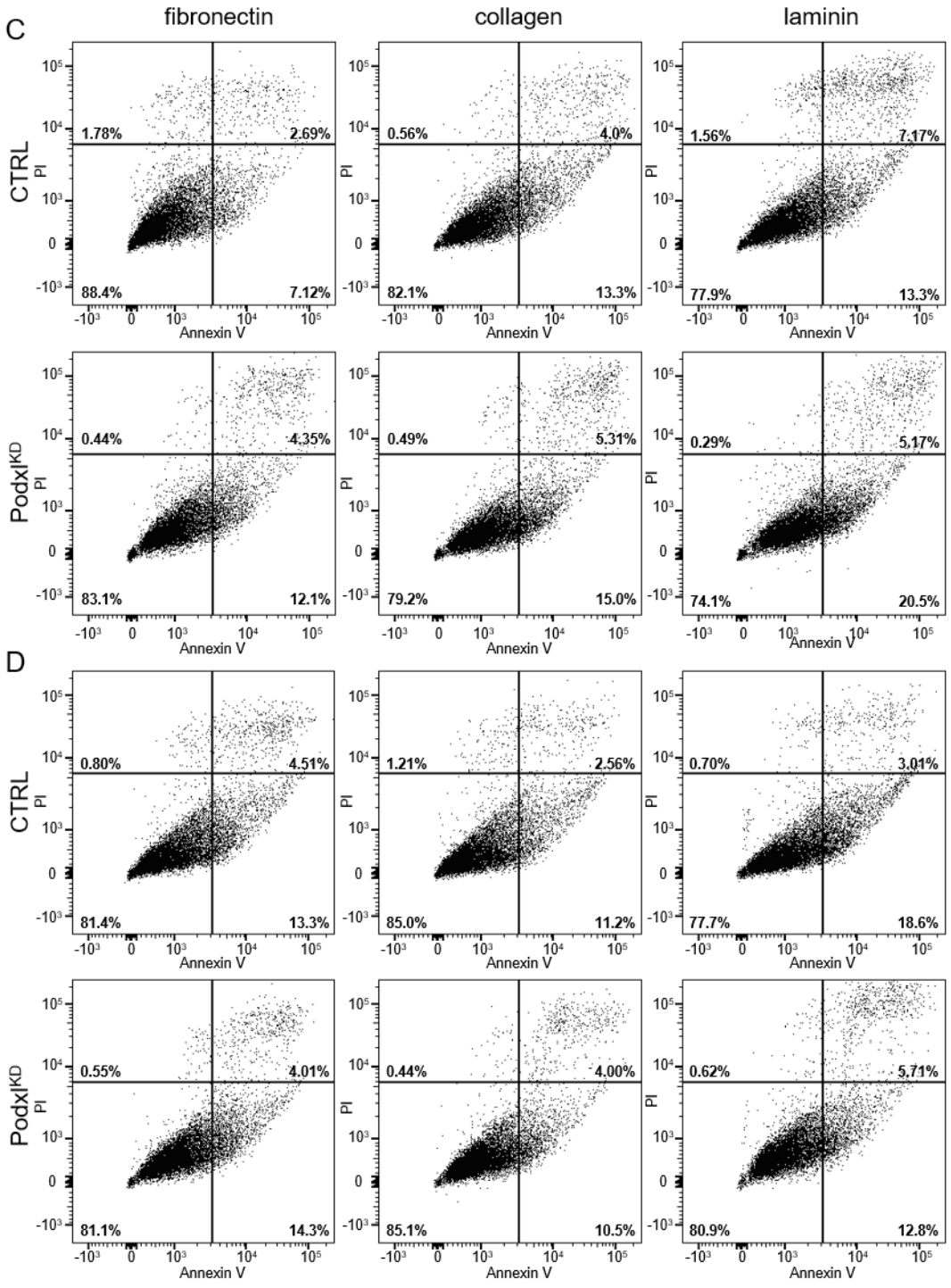


Fig. S3. Podocalyxin expression does not affect cell viability in HUVEC cultures. Propidium iodide (PI) and Annexin V flow cytometry apoptosis assay of CTRL and PODXL^{KD} HUVEC cells plated on fibronectin, collagen and laminin for **(A)** 24 hours, **(B)** 48 hours, **(C)** 72 hours and **(D)** 96 hours.

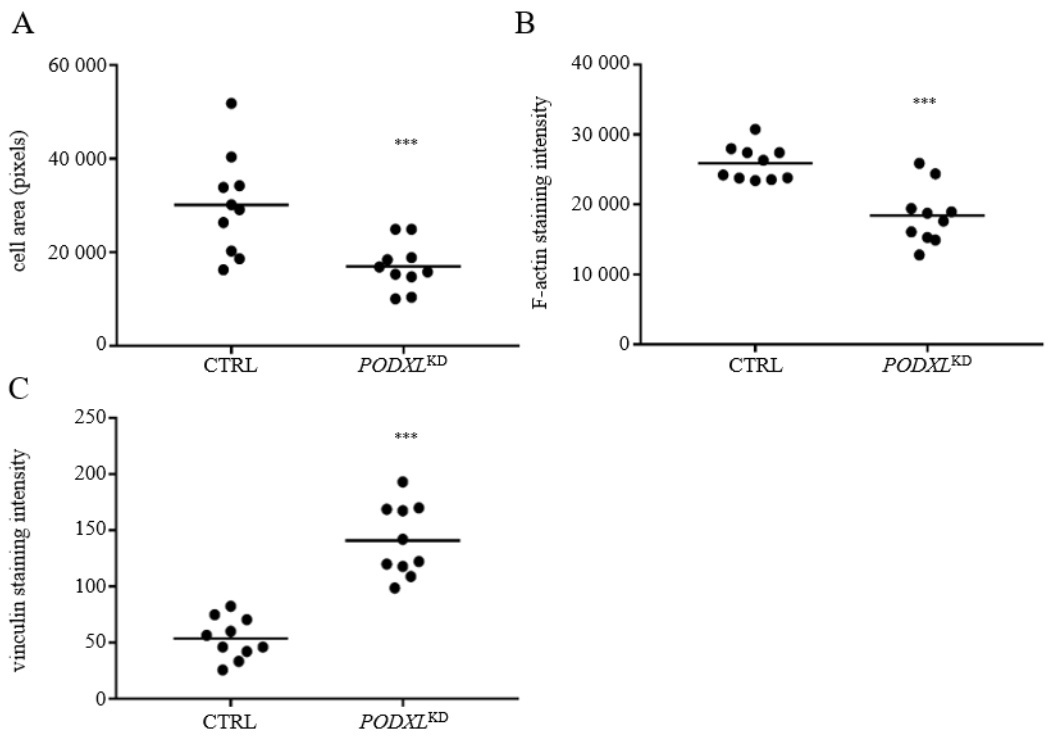


Fig. S4. Podocalyxin expression in HUVEC is required for normal cell spreading, F-actin and vinculin levels. CTRL and PODXL^{KD} HUVEC 72 h after seeding. Data shown are representative of three independent experiments. n=10 cells per experiment. 10 cells measured in 2 independently stained wells. **(A)** ImageJ quantification of cell area. Plated on laminin. **(B)** ImageJ quantification of F-actin staining intensity. Plated on laminin. **(C)** ImageJ quantification of vinculin staining intensity. Plated on fibronectin. ***Significantly different than control with $p<0.0001$ (determined by Student's t-test).

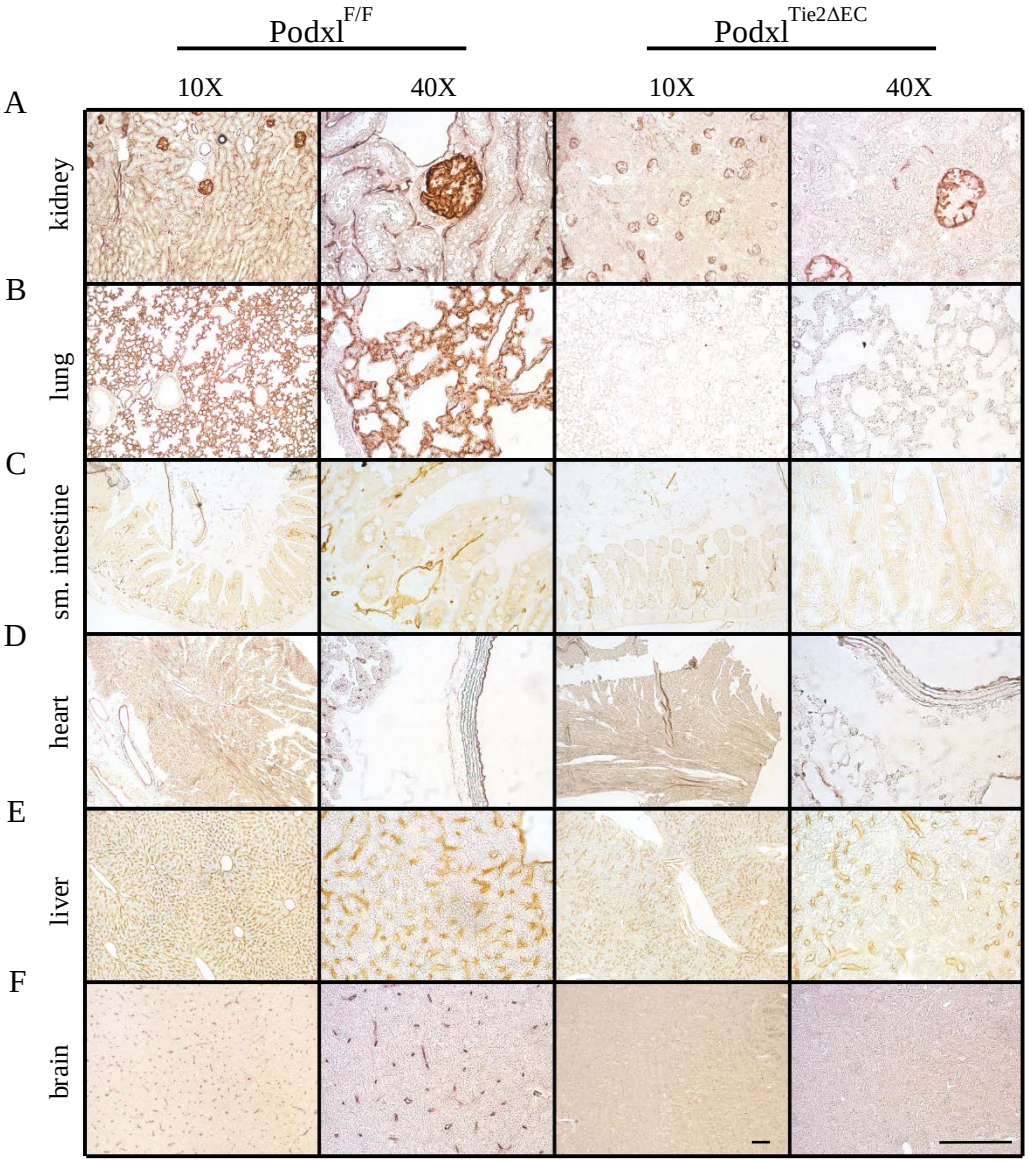


Fig. S5. Tie2Cre drives efficient deletion of podocalyxin in multiple organs. 3,3′-diaminobenzidine (DAB) immunohistochemical staining of podocalyxin. **(A)** Specific loss of podocalyxin in kidney microvessels and glomerular endothelia. Podocalyxin expression is retained in kidney podocytes. **(B)** Complete loss of podocalyxin protein in lung microvessels. **(C)** Complete loss of podocalyxin protein in the small intestine. **(D)** Patchy deletion of podocalyxin in heart ventricles. **(E)** Patchy deletion of podocalyxin in liver sinusoidal endothelia. **(F)** Complete loss of podocalyxin protein in brain microvessels. Scale bars = 100 µm.

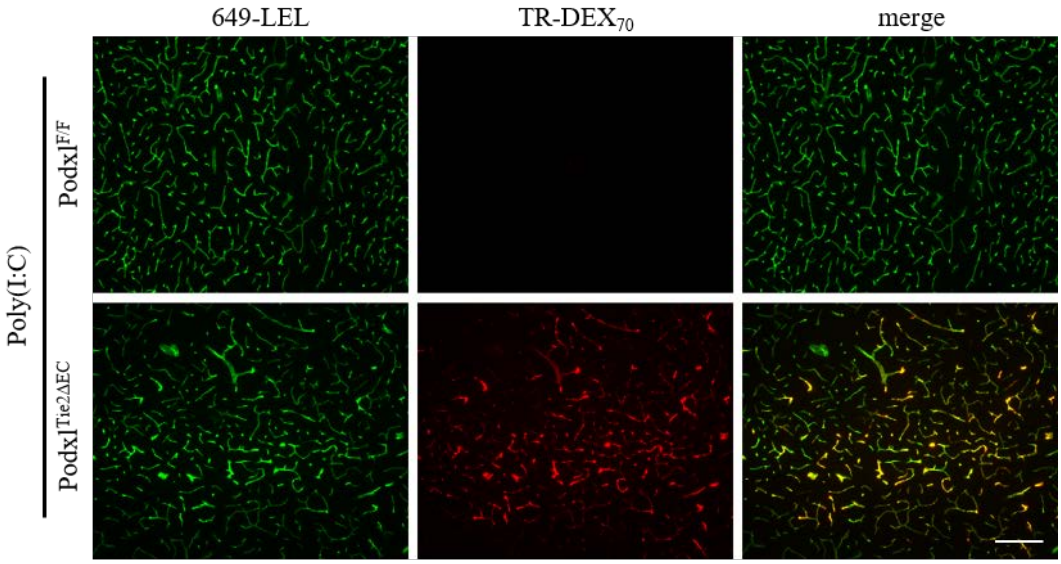


Fig. S6. Podocalyxin expression in vascular endothelia promotes barrier function during Poly(I:C) -induced inflammation. Fluorescent micrographs of the cerebral cortex region of brain harvested from naïve and LPS-treated (5 mg/kg i.p. for 6 h) mice. 10 min before sacrifice 649-LEL and TR-DEX_{70} was circulated via retro-orbital route to label vessel lumens and assess vascular barrier function. Scale bars = 100 µm.

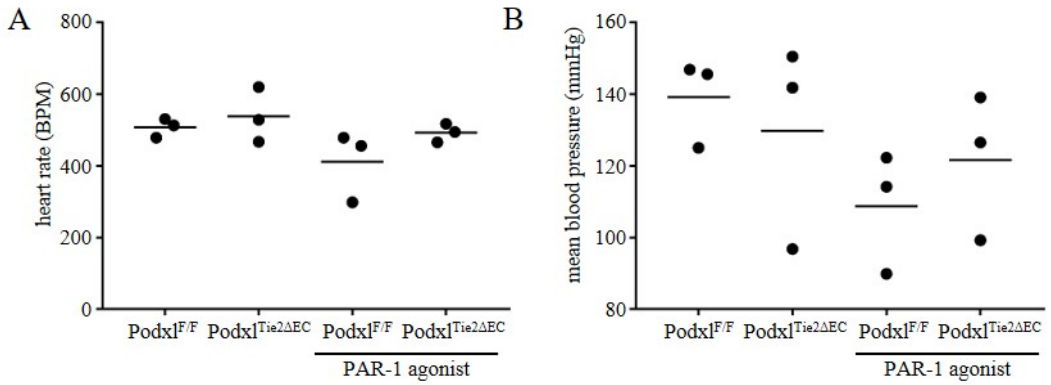


Fig. S7. Treatment with a PAR-1 agonist does not alter blood pressure or heart rate. Heart rate and blood pressure as measured by CODA noninvasive blood pressure system **(A)** Average heart rate (beats per minute (BPM)) of $Podxl^{F/F}$ and $Podxl^{Tie2\Delta EC}$ mice after treatment with LPS and after administration of a PAR-1 agonist. n=3. **(B)** Mean blood pressure (mmHg) readings of $Podxl^{F/F}$ and $Podxl^{Tie2\Delta EC}$ mice after treatment with LPS and after administration of a PAR-1 agonist. n=3. Data points represent measurements from individual mice and the horizontal lines represent means. Differences not significant as determined by Student's t-test.

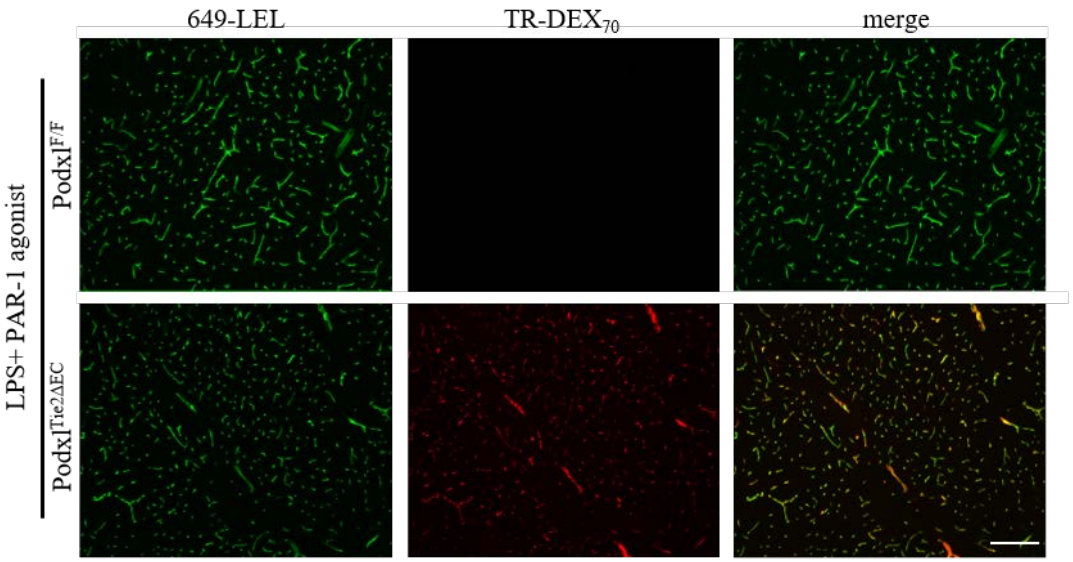


Fig. S8. Injection of a PAR-1 agonist does not alter barrier function after LPS-induced inflammation. Fluorescent micrographs of the cerebral cortex region of brain harvested from LPS (5 mg/ kg i.p. for 6 h) and PAR-1 agonist (5 mg/ kg i.v.)-treated mice. 10 min before sacrifice 649-LEL and TR-DEX_{70} was circulated via retro-orbital route to label vessel lumens and assess vascular barrier function. Scale bars = 100 µm.

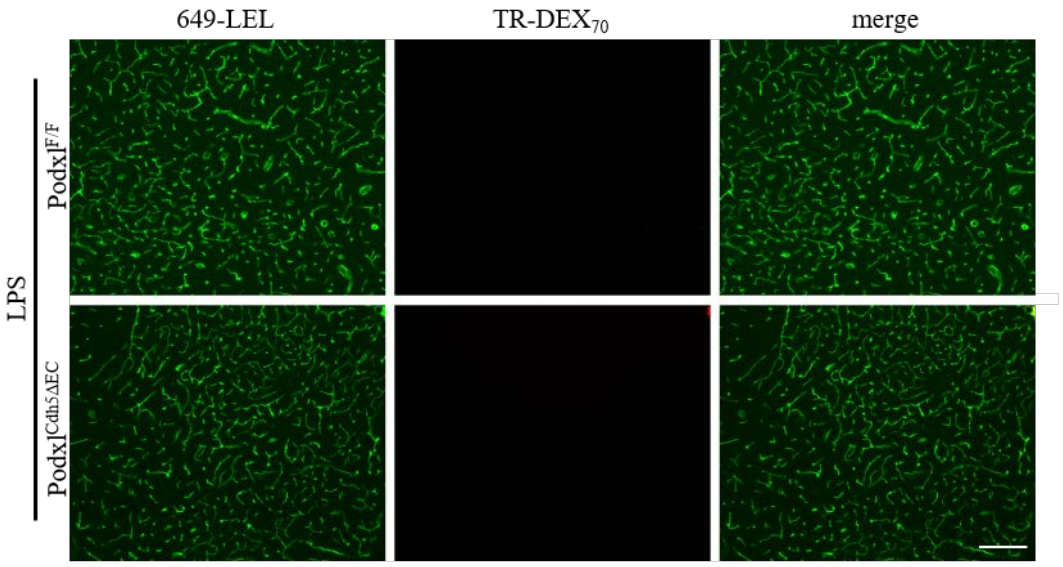


Fig. S9. Treatment of Podxl$^{Cdh5\Delta EC}$ mice with LPS induces no vascular permeability in the brain. Fluorescent micrographs of the cerebral cortex region of brain harvested from naïve and LPS-treated (5 mg/kg i.p. for 6 h) mice. 10 min before sacrifice 649-LEL and TR-DEX$_{70}$ was circulated via retro-orbital route to label vessel lumens and assess vascular barrier function. Scale bars = 100 µm.

Captions for movies

Movie S1. Podocalyxin promotes endothelial monolayer maintenance. Live phase-contrast video microscopy of WT HUVEC monolayers plated on fibronectin measured by IncuCyte ZOOM™ software over 96 h time course.

Movie S2. Podocalyxin promotes endothelial monolayer maintenance. Live phase-contrast video microscopy of PODXL^{KD} HUVEC monolayers plated on fibronectin measured by IncuCyte ZOOM™ software over 96 h time course.

Movie S3. Podocalyxin promotes endothelial monolayer maintenance. Live phase-contrast video microscopy of WT HUVEC monolayers plated on gelatin measured by IncuCyte ZOOM™ software over 96 h time course.

Movie S4. Podocalyxin promotes endothelial monolayer maintenance. Live phase-contrast video microscopy of PODXL^{KD} HUVEC monolayers plated on gelatin measured by IncuCyte ZOOM™ software over 96 h time course.

Movie S5. Podocalyxin promotes endothelial monolayer maintenance. Live phase-contrast video microscopy of WT HUVEC monolayers plated on laminin measured by IncuCyte ZOOM™ software over 96 h time course.

Movie S6. Podocalyxin promotes endothelial monolayer maintenance. Live phase-contrast video microscopy of PODXL^{KD} HUVEC monolayers plated on laminin measured by IncuCyte ZOOM™ software over 96 h time course.

Movie S7. Video observation of behavioral changes observed after administration of LPS and PAR-1 agonist. Video analysis started immediately after i.v. injection with PAR-1 agonist (6 h post LPS administration). Two subsequent mice were observed in the video; one WT (t = 0 sec) one *Podxl*$^{\Delta Tie2Cre}$ (t = 58 sec) respectively. Activity was scored by reviewing videos based on total motion observed at 10 s intervals (with 1=normal, 0.5=reduced activity and 0=no activity) and time for full recovery (sustained return to score = 1).

Movie S8. Video observation of behavioral changes observed after administration of poly(I:C) and PAR-1 agonist. Video analysis started immediately after i.v. injection with PAR-1 agonist (6 h post poly(I:C) administration). Two mice were observed subsequently in the video; one WT (t = 0 sec) one *Podxl*$^{\Delta Tie2Cre}$ (t = 58 sec) respectively. Activity was scored by reviewing videos based on total motion observed at 10 s intervals (with 1=normal, 0.5=reduced activity and 0=no activity) and time for full recovery (sustained return to score = 1).