Supplemental Data
Mechanosensation by endothelial PIEZO1 is required for leukocyte diapedesis
(Wang et al.)

Supplemental Figures

Suppl. Fig. 1

Suppl. Figure 1. Effect of endothelial loss of PIEZO1 on various basal parameters in vitro and in vivo. (A,B) Expression of Piezo1 in endothelial cells of wild-type and EC-Piezo1-KO mice. (A) Endothelial cells were isolated from lungs of wild-type or EC-Piezo1-KO mice, and Piezo1 mRNA was analyzed by RT-PCR. The bar diagram shows the statistical analysis (n= 4 in each group). (B) Effect of tamoxifen treatment of Tie2-CreER<sup>T2</sup>;Piezo1<sup>flox/flox</sup> mice on Piezo1 protein levels in lung endothelial cells as analyzed by Western blotting. 7 days after
tamoxifen treatment, endothelial cells were isolated and lysates were analyzed by immunoblotting using anti-Piezo1 antibodies. Shown are data from 2 different animals. (C) HUVECs were transfected with control siRNA or siRNA directed against PIEZO1 and were incubated with 10 ng/ml TNFα for 15 h. Cells were then lysed and the indicated proteins were analyzed by immunoblotting using the indicated antibodies. (D) Total number of neutrophils per area in sections of ears from wild-type and EC-Piezo1-KO mice treated with croton oil (n = 5 mice, 12 images per animal were analyzed). (E-I) EC-Piezo1-KO and control mice were analyzed by intravital microscopy of Cremaster venules 4 hours after injection of 50 ng IL1β for the number of adhering PMNs (E), rolling PMNs per second per mm of vessel length (F), rolling flux fraction (G), Newtonian wall shear rates (H) and wall shear rates (I) (n=9 animals per group). (J,K) Lung endothelial cells were isolated from wild-type (WT) and EC-Piezo1-KO mice and analyzed by RNA-sequencing (J) or by immunoblotting (K). Shown are mean values ± s.e.m.; *, P ≤ 0.05; n.s., not significant (unpaired t-test).
Suppl. Figure 2. Regulation of [Ca^{2+}]_i and PIEZO1 channel activity by flow and leukocytes. (A) HUVECs were pre-activated with TNFα, loaded with Fura-2 and were then exposed to PMNs alone, to low flow (1.2 dynes/cm^2) alone or to both. [Ca^{2+}]_i was determined
by ratiometric imaging. 5 representative traces are shown per condition. Bar diagram shows the area under the curve (AUC) of the \([\text{Ca}^{2+}]\); traces from 4 independent experiments (20-40 cells were analyzed per experiment) (au, arbitrary units). The arrow indicates the time point of PMN addition. (B) HUVECs were preactivated with TNF\(\alpha\), loaded with Fluo-4, and were then exposed to PMNs. Thereafter, different flow rates (0.4, 0.8 and 1.2 dynes/cm\(^2\)) were given and \([\text{Ca}^{2+}]\) was determined as fluorescence intensity (RFU, relative fluorescence units). The arrow indicates the time point of PMN addition. Shown is a representative experiment. The bar diagram shows the area under the curve (AUC) of the \([\text{Ca}^{2+}]\); traces from 3 independent experiments (15-35 cells were analyzed per experiment) (a.u., arbitrary units). (C) HUVECs loaded with Fluo4 were exposed to human neutrophils together with flow (1.2 dynes/cm\(^2\)), and intracellular free \([\text{Ca}^{2+}]\) was measured during different phases of PMN-endothelial cell interaction (n=6 per group) (RFU, relative fluorescence units). (D) HUVECs transfected with control (siCtrl) or PIEZO1-specific siRNA (siPIEZO1) were preactivated with TNF\(\alpha\), loaded with Fura-2 and were then exposed to PMNs and low flow (1.2 dynes/cm\(^2\)) given together. \([\text{Ca}^{2+}]\) was determined by ratiometric imaging. Shown is a representative experiment. The bar diagram shows the area under the curve (AUC) of the \([\text{Ca}^{2+}]\); traces from 4 independent experiments (15-35 cells were analyzed per experiment) (a.u., arbitrary units). (E,F) Untransfected HUVECs were preactivated with TNF\(\alpha\), loaded with Fluo-4 and pretreated without or with 10 \(\mu\)M BAPTA-AM (E) or 50 \(\mu\)M EGTA (F). \([\text{Ca}^{2+}]\) was removed from the medium in (F). Cells were then exposed to both PMNs and low flow (1.2 dynes/cm\(^2\)) given together. \([\text{Ca}^{2+}]\) was determined as fluorescence intensity (RFU, relative fluorescence units) or by ratiometric imaging. Shown are representative experiments. The bar diagrams show the area under the curve (AUC) of the \([\text{Ca}^{2+}]\); traces from 3 independent experiments (15-30 cells were analyzed per experiment) (a.u., arbitrary units). (G,H) Currents from MLECs of wild-type (Control) or EC-Piezo1-KO mice were recorded at a holding potential of −80 mV in the whole cell patch clamp configuration. MLECs were exposed to 1 \(\mu\)M Yoda1, or to mechanical stimulation using a fire-polished glass pipette (Poking). 5 \(\mu\)M GsMTx4 was applied to cells as indicated. (n = 6-9 independent measurements per condition). Shown are mean values ± s.e.m.; *, \(P \leq 0.05\); **, \(P \leq 0.01\); ***, \(P \leq 0.001\) (one-way ANOVA (A,B,H), unpaired t-test (C-F)).
Suppl. Figure 3. Increases in [Ca\textsuperscript{2+}]\textsubscript{i} and downstream signaling mediated by PIEZO1 and ICAM-1. (A,B) HUVECs were transfected with control siRNA or with siRNA directed against ICAM-1, and cells were left untreated (A) or were treated in the absence or presence of TNF\textsubscript{α} (B). Thereafter, expression of ICAM-1 was determined by qRT-PCR (A) or by immunoblotting using an anti-ICAM-1 antibody (B). GAPDH served as a control. Shown is the statistical evaluation of 3 independently performed experiments (A) or a representative of 3 independently performed experiments (B). (C) HUVECs were loaded with Fluo4 and exposed to low flow (1.2 dynes/cm\textsuperscript{2}) alone, anti-ICAM-1 antibody-linked beads (beads) or both flow and beads, and free [Ca\textsuperscript{2+}]\textsubscript{i} was determined (RFU, relative fluorescence units). The bar diagram shows the statistical evaluation of the area under the curve (AUC) (n=3 independent experiments) (a.u., arbitrary units). (D,E) TNF\textsubscript{α}-activated HUVECs were exposed to low flow alone, to control beads alone or to both low flow and control beads. Thereafter, the free [Ca\textsuperscript{2+}]\textsubscript{i} was determined after loading of HUVECs with Fluo-4 (D) or immunoblot analysis of total and phosphorylated PYK2, SRC and MLC (E) was performed. The bar diagram (D) shows the area under the curve (AUC) or the [Ca\textsuperscript{2+}]\textsubscript{i} traces from 3 independent experiments (a.u., arbitrary units). The bar diagram in E shows the densitometric analysis of 3 independently performed experiments.
experiments. (F-I) Schematic of antibody-induced clustering of ICAM-1 (F). HUVECs transfected with control siRNA (siCtrl) or siRNA directed against *ICAM-1* or *PIEZO1* were activated with TNFα and were then exposed to low flow (1.2 dynes/cm²) and anti-ICAM-1 clustering antibodies (ICAM-1 XL) given together (G) or to low flow and anti-ICAM-1 clustering antibodies alone or given together (H,I). Thereafter, the free [Ca²⁺] was determined after loading of cells with Fluo-4 (G). The bar diagram shows the area under the curve (AUC) of the [Ca²⁺] traces from 3 independent experiments (a.u., arbitrary units). Alternatively, immunoblot analysis of total and phosphorylated of PYK2, SRC and MLC was performed. Immunoblot analysis of GAPDH served as control (H,I). The bar diagram shows the densitometric analysis of 3 independent experiments. Shown are mean values ± s.e.m.; *P ≤ 0.05; **P ≤ 0.01; n.s., non-significant (unpaired t-test (A) or one-way ANOVA (C-I)).
Suppl. Figure 4. Schematic diagram depicting principle of membrane tension probes. FliptR\(^1\) (A) and MSS\(^2\) (B). The fluorescent membrane tension probe FliptR was described by Colom et al. and allows the monitoring of changes in membrane tension by fluorescence lifetime imaging microscopy \(^1\). The MSS biosensor was described by Li et al. and allows to determine alterations of cell membrane tension by measuring fluorescence resonance energy transfer (FRET) \(^2\). The MSS biosensor contains a linker sequence (GPGGA)\(_8\), which is inserted between two fluorescent proteins, enhanced cyan fluorescence protein (ECFP) and yellow fluorescent protein for energy transfer (YPet). The YPet is linked to 21 amino acids from Lyn kinase, and ECFP is linked to 14 amino acids from K-Ras, which link the tension sensor to phospholipids in the region of lipid raft and non-lipid raft, respectively \(^2\). When force extends the elastic linker, FRET efficiency decreases, otherwise it increases.
Suppl. Fig. 5

**Suppl. Figure 5.** Analysis of knock-down efficiency in HUVECs. HUVECs were transfected with control siRNA (siCtrl) or siRNA directed against *ACTN4* (A) or *CTTN* (B). Knock-down efficiency was analyzed by qRT-PCR (n=3 independent experiments). Shown are mean values ± s.e.m.; **P ≤ 0.01 (unpaired t-test).
Supplemental Table

**Supplemental Table 1.**

HUVECs pretreated with 10 ng/ml TNFα were transfected with 360 siRNAs pools against RNAs encoding transmembrane proteins expressed in endothelial cells and were then exposed to THP-1 monocytic cells for 3 hours. Shown is the log ratio of THP-1 cells which transmigrated the HUVEC monolayer transfected with a particular siRNA pool and with control siRNA.

| Gene     | Mean | SEM | Gene     | Mean | SEM | Gene     | Mean | SEM |
|----------|------|-----|----------|------|-----|----------|------|-----|
| ESAM     | -1.42| 0.23| ATP13A1  | -0.37| 0.37| ADAM10   | 0    | 0.63|
| ALCAM    | -1.33| 0.12| TSPAN18  | -0.37| 0.40| ITGB5    | 0    | 0.06|
| PIEZO1   | -1.21| 0.16| PLXNA4   | -0.37| 0.26| SLC39A10 | 0.01 | 0.52|
| TEMEM63B | -0.99| 0.15| CX3CL1   | -0.37| 0.38| TSPAN8   | 0.01 | 0.06|
| ICAM1    | -0.86| 0.26| BCAM     | -0.37| 0.18| SLC12A2  | 0.01 | 0.42|
| PVR      | -0.84| 0.16| PLAUR    | -0.37| 0.31| SLC4A7   | 0.02 | 0.23|
| EMP2     | -0.82| 0.16| STT3B    | -0.36| 0.32| SERINC3  | 0.02 | 0.60|
| ENG      | -0.79| 0.15| F2R      | -0.36| 0.24| EMCN     | 0.02 | 0.16|
| NRP2     | -0.78| 0.21| TMEM126A | -0.36| 0.34| PTGFRN   | 0.03 | 0.26|
| ICAM2    | -0.76| 0.37| RNF13    | -0.35| 0.22| SSR1     | 0.04 | 0.19|
| MCAM     | -0.75| 0.56| CD47     | -0.34| 0.26| TGOLN2   | 0.04 | 0.62|
| SPNS2    | -0.75| 0.32| NCSTN    | -0.34| 0.17|TMEM109   | 0.05 | 0.13|
| GNAQ     | -0.74| 0.18| ADIPO1   | -0.34| 0.28| SLC7A1   | 0.05 | 0.29|
| TMEM9    | -0.74| 0.21| CD63     | -0.34| 0.15| APP      | 0.05 | 0.08|
| EFNA4    | -0.74| 0.28| ATP1B3   | -0.34| 0.14| DDR2     | 0.06 | 0.21|
| SLC35A5  | -0.73| 0.07| CA4      | -0.33| 0.50| FAM171A1 | 0.06 | 0.49|
| TMEM159  | -0.73| 0.18| SLC29A1  | -0.33| 0.20| RPN1     | 0.06 | 0.87|
| PLXNB2   | -0.73| 0.09| NPR1     | -0.33| 0.29| TMEM248  | 0.07 | 0.15|
| FGFR3    | -0.72| 0.36| SLC16A1  | -0.33| 0.16| MFAP3    | 0.07 | 0.20|
| NOTCH2   | -0.71| 0.46| TMEM47   | -0.33| 0.44|TMEM183A  | 0.07 | 0.29|
| GPR4     | -0.71| 0.07| RYK      | -0.33| 0.29| ECE1     | 0.08 | 0.26|
| TIE1     | -0.71| 0.13| DCBLD1   | -0.32| 0.48| PLXND1   | 0.09 | 0.42|
| CD44     | -0.69| 0.14| PTPRA    | -0.32| 0.14| MYOF     | 0.1  | 0.47|
| ANPEP    | -0.69| 0.22| TSPAN13  | -0.32| 0.34| GHR      | 0.1  | 0.32|
| P2RY2    | -0.69| 0.31| CD36     | -0.31| 0.33|TMEM123   | 0.11 | 0.16|
| GJA1     | -0.68| 0.12| TM9SF2   | -0.31| 0.46|TMEM259   | 0.12 | 0.47|
| CD81     | -0.68| 0.19| SERINC1  | -0.31| 0.10| STIM1    | 0.12 | 0.21|
| KDR      | -0.67| 0.09| IGF2R    | -0.31| 0.20| TSPAN15  | 0.13 | 0.39|
| NRP1     | -0.67| 0.10| SLC44A1  | -0.31| 0.18| TMEM98   | 0.13 | 0.04|
| ADGRF5   | -0.67| 0.21| F11R     | -0.31| 0.24|TMEM140   | 0.13 | 0.06|
| FAT4     | -0.66| 0.18| ADRB2    | -0.3  | 0.09| DCHS1    | 0.14 | 0.12|
| GPR108   | -0.66| 0.32| MXRA8    | -0.3  | 0.07| FGFR1    | 0.14 | 0.17|
| HFE      | -0.66| 0.23| TTYH3    | -0.3  | 0.06| ITGAV    | 0.15 | 0.27|
| SUSD1    | -0.65| 0.38| SLC44A2  | -0.3  | 0.10| SLC6A6   | 0.17 | 0.14|
| Gene       | p-value | p-value |
|------------|---------|---------|
| LMBR1L     | 0.64    | 0.18    |
| LRP6       | 0.64    | 0.08    |
| TEMEM147   | 0.64    | 0.07    |
| FURIN      | 0.63    | 0.13    |
| TEMEM231   | 0.63    | 0.18    |
| PTPRK      | 0.62    | 0.23    |
| DLL1       | 0.62    | 0.23    |
| SLCO3A1    | 0.61    | 0.25    |
| ANTXR2     | 0.61    | 0.28    |
| MNSC1      | 0.61    | 0.38    |
| ATRN       | 0.61    | 0.07    |
| TMEM39A    | 0.6     | 0.08    |
| ADAM9      | 0.6     | 0.13    |
| TEMEM245   | 0.6     | 0.17    |
| TGFBR1     | 0.6     | 0.15    |
| TPCN1      | 0.6     | 0.22    |
| HLA-DMA    | 0.6     | 0.13    |
| ATP2B4     | 0.59    | 0.28    |
| IL27RA     | 0.59    | 0.10    |
| ITG4A      | 0.58    | 0.21    |
| SLC30A1    | 0.57    | 0.16    |
| ERM1       | 0.57    | 0.13    |
| SL2C1A1    | 0.56    | 0.11    |
| SCAP       | 0.56    | 0.16    |
| ADAM17     | 0.55    | 0.18    |
| SCN5A      | 0.55    | 0.33    |
| TLR3       | 0.55    | 0.37    |
| PTTPRU     | 0.55    | 0.21    |
| LTBR       | 0.55    | 0.32    |
| ADRG1      | 0.55    | 0.43    |
| EDNRB      | 0.55    | 0.35    |
| CXCL16     | 0.54    | 0.35    |
| HRH1       | 0.53    | 0.18    |
| FLT4       | 0.53    | 0.37    |
| PCDHG5     | 0.52    | 0.05    |
| SLC2A1     | 0.52    | 0.13    |
| SLC39A6    | 0.52    | 0.18    |
| NIPAL2     | 0.51    | 0.11    |
| ADRB1      | 0.51    | 0.08    |
| ATP13A2    | 0.51    | 0.23    |
| LMBRD1     | 0.51    | 0.08    |
| CD109      | 0.51    | 0.53    |
| PCDH17     | 0.51    | 0.31    |
| BACE2      | 0.5     | 0.42    |
| EFNBI      | 0.49    | 0.08    |
| Gene      | Adj. p1  | Adj. p2  | Adj. p3  | Adj. p4  | Adj. p5  |
|-----------|----------|----------|----------|----------|----------|
| SPPL2A    | -0.49    | 0.63     | PLXNA2   | -0.17    | 0.26     | RELL1    | 0.88     | 0.10     |
| LRP5      | -0.49    | 0.19     | CALCRL  | -0.16    | 0.16     | TMEM115  | 0.7      | 0.08     |
| ITGB3     | -0.48    | 0.23     | CPD     | -0.16    | 0.22     | TGFB3    | 0.71     | 0.14     |
| ABCA1     | -0.48    | 0.12     | HYAL2   | -0.15    | 0.11     | EBP      | 0.73     | 0.16     |
| SGC8      | -0.48    | 0.13     | TNFRSF14| -0.15    | 0.07     | DAG1     | 0.74     | 0.24     |
| CLEC1A    | -0.48    | 0.32     | TSPAN7  | -0.15    | 0.06     | SLC43A3  | 0.78     | 0.15     |
| MYADM     | -0.47    | 0.11     | PSEN1   | -0.14    | 0.47     | LRIG3    | 0.78     | 0.06     |
| TMEM184C  | -0.47    | 0.12     | TMEM184B| -0.14    | 0.24     | ATG9A    | 0.81     | 0.39     |
| TMEM230   | -0.47    | 0.14     | CD164   | -0.14    | 0.50     | NECTIN2  | 0.85     | 0.68     |
| TMEM63A   | -0.47    | 0.15     | TM9SF3  | -0.13    | 0.17     | TMEM222  | 0.87     | 0.26     |
| TM9SF1    | -0.47    | 0.16     | SLC1A5  | -0.13    | 0.31     | PCDHB13  | 0.87     | 0.51     |
| LYVE1     | -0.47    | 0.18     | MPZL1   | -0.13    | 0.22     | TMEM219  | 0.91     | 0.45     |
| UBAC2     | -0.46    | 0.34     | TMEM165 | -0.13    | 0.36     | JAM2     | 0.96     | 0.35     |
| SLC31A1   | -0.46    | 0.16     | ITFG1   | -0.13    | 0.29     | TMEM208  | 0.97     | 0.78     |
| TMEM9B    | -0.46    | 0.16     | LRRC32  | -0.12    | 0.09     | APLNR    | 1.03     | 0.45     |
| FLRT2     | -0.46    | 0.08     | TSPAN9  | -0.11    | 0.10     | ITGA6    | 1.05     | 0.34     |
| TMEM100   | -0.46    | 0.16     | TMEM43  | -0.11    | 0.64     | NAGPA    | 1.07     | 0.30     |
| PMEPA1    | -0.45    | 0.43     | PCDHA10 | -0.1     | 0.13     | SEMA6D   | 1.12     | 0.23     |
| SLC39A14  | -0.45    | 0.32     | ECSCR   | -0.1     | 0.07     | PSEN2    | 1.23     | 0.23     |
| CXCR4     | -0.44    | 0.06     | LRIG1   | -0.09    | 0.16     | OPN3     | 1.25     | 0.42     |
| TMCO3     | -0.44    | 0.08     | TMEM258 | -0.09    | 0.08     | SIGIRR   | 1.32     | 0.30     |
| DGRG2     | -0.44    | 0.08     | PCDH12  | -0.09    | 0.41     | RHBD2    | 1.4      | 0.31     |
| SEMA6A    | -0.44    | 0.13     | THSD1   | -0.08    | 0.11     | ITM2B    | 1.45     | 0.13     |
| TMEM64    | -0.44    | 0.17     | C5orf15 | -0.07    | 0.32     | PKD1     | 1.56     | 0.42     |
| TMEM88    | -0.44    | 0.13     | ATP1A1  | -0.07    | 0.09     | NIPAL3   | 1.66     | 0.16     |
| ITM2C     | -0.43    | 0.13     | BMP2    | -0.07    | 0.21     | PIK3IP1  | 1.7      | 0.45     |
| NRCAM     | -0.43    | 0.14     | TMEM106B| -0.07    | 0.21     | GPRC5A   | 1.7      | 0.06     |
| SYPL1     | -0.43    | 0.41     | ANO6    | -0.06    | 0.23     | JAG2     | 1.79     | 0.21     |
| ZDHHC5    | -0.42    | 0.10     | EPHA2   | -0.05    | 0.23     | HBEGF    | 1.81     | 0.56     |
| TM7SF3    | -0.42    | 0.25     | TM9SF4  | -0.04    | 0.16     | TMEM181  | 1.82     | 0.27     |
| CD82      | -0.41    | 0.11     | CD200   | -0.04    | 0.20     | MMP15    | 1.83     | 0.16     |
| SCARB2    | -0.41    | 0.25     | NECTIN3 | -0.03    | 0.10     | IFNAR1   | 1.91     | 0.15     |
| CLSTN1    | -0.4     | 0.08     | ADGRL2  | -0.03    | 0.05     | CASD1    | 1.92     | 0.26     |
| CSF1      | -0.4     | 0.15     | SLC9A1  | -0.03    | 0.28     | CD9      | 2.11     | 0.39     |
| TFRA1     | -0.4     | 0.07     | GINM1   | -0.03    | 0.19     | JAM3     | 2.28     | 0.23     |
| EMP1      | -0.39    | 0.11     | ADIPOR2 | -0.03    | 0.08     | SORT1    | 2.41     | 0.31     |
| CCRL2     | -0.39    | 0.16     | DPP4    | -0.02    | 0.29     | PTPRB    | 2.54     | 0.29     |
| GJA4      | -0.39    | 0.38     | PCNX1   | -0.02    | 0.09     | TEK      | 2.55     | 0.27     |
| KIT       | -0.38    | 0.16     | CD9     | -0.01    | 0.26     | TMEM2    | 2.58     | 0.53     |
| SLC5A3    | -0.38    | 0.20     | TSPAN6  | -0.01    | 0.24     | CDH5     | 2.61     | 0.12     |
| SLC38A2   | -0.37    | 0.40     | CPM     | 0        | 0.19     | SLC39A9  | 2.63     | 0.41     |
Additional Methods

Cell culture and cell isolation

Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza and cultured with EGM-2 (Lonza) supplemented with 5% fetal bovine serum (FBS) (Lonza). Only confluent cells at passage ≤4 were used in experiments. THP-1 monocyte cells were obtained from Sigma (Cat. No. 88081201) and were cultured in RPMI 1641 medium (Invitrogen) supplemented with 2 mM glutamine and 10% FBS. The bEnd.3 cell line was obtained from ATCC and cells were cultured in DMEM medium (Invitrogen) supplemented with 10% FBS. Primary mouse lung endothelial cells (MLECs) were isolated using CD31-labelled dynabead (Miltenyi Biotec, Cat. No. 130-097-418) and were further purified by FACS using anti-CD144-PE antibodies (BD Biosciences, Cat. No. 562243) as described previously. Human polymorphonuclear leukocytes (PMNs) and peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood by density gradient centrifugation using Histopaque 1077 and 1119 (Sigma-Aldrich) and were resuspended in Hanks balanced salt solution (HBSS) with 20 mM HEPES (pH 7.4) and 0.5% human serum albumin (Sigma-Aldrich). Mouse PMNs from murine femora and tibiae were isolated with an EasySep Mouse Neutrophil Enrichment Kit (STEMCELL Technologies, Canada) according to manufacturer’s instructions. The PMNs were resuspended in Hanks balanced salt solution (HBSS) containing 20 mM HEPES (pH 7.4) and 0.5% fetal calf serum and were immediately used for transmigration assays.

siRNA-mediated knockdown

Cells were transfected with siRNAs using Opti-MEM and Lipofectamine RNAiMAX (Invitrogen) as described previously. For transfection of cells in ibidi flow chambers, 20 pmoles of siRNA were mixed gently with RNAiMAX in 20 μl Opti-MEM (Thermo
Fisher) and incubated for 30 minutes at room temperature. The mixture was then added to 100 μl of cell culture medium, and cells in the flow chamber (ibidi μ-slide I) were covered by the medium. The medium was changed after 6 hours. 18 hours later, the siRNA transfection was repeated, and experiments were performed 48 hours later. For transfection of cells in 96-transwell plates (Corning), 2.5 pmoles siRNA in 10 μl were prepared and added to 50 μl cell culture medium. siRNAs against PIEZO1 and ICAM1 were from Sigma-Aldrich, and siRNAs against ACTN4 and CTTN were from Genepharma. The targeted sequences of siRNAs directed against RNAs encoding PIEZO1, ICAM-1, α-actinin-4 and cortactin were: PIEZO1 (human), 5′-CCAAGTACTGGATCTATGT-3′, 5′-GCAAGTTCGTGCGCGGATT-3′, and 5′-AGAAGAAGATCGTCAAGTA-3′; ICAM-1 (human), 5′-CAGCGGAAGATCAAGAAAT-3′, 5′-CCGAGCTCAAGTGTCTAAA-3′, 5′-CAACCAATGTGCTATTCAA-3′; α-actinin-4 (human) 5′-CCACATCAGCTGGAGGATGGTC-3′, 5′-GCAGCAAGCGCGACAGCTTC-3′; cortactin (human) 5′-CCAGGAGCATATCAACATA-3′; 5′-GCAACTTATTGTATCTGAA-3.

SiRNAs used for the screen were pools of siRNAs of a customized siRNA library (Sigma) directed against 360 genes encoding transmembrane proteins enriched in HUVECs. Only siRNAs resulting in suppression of expression levels to less than 25% of control levels as determined by quantitative RT-PCR were used.

**In vitro transmigration assay**

HUVECs or MLECs were seeded at 1.5 × 10^4 cells in 100 μl and were cultured on collagen-coated 96-transwell plates with polyester membranes of 8 μm pore size (Corning) until reaching confluency and were then incubated with 10 ng/ml recombinant TNFα (PeproTech, Cat. No. 300-01A) for 16 hours prior to the assay. For transmigration experiments, the medium of the upper compartment was removed and
$8 \times 10^3$ calcein-AM-labelled PMNs were added in 50 μl of HBSS alone or in the presence of the indicated substances. 30 min later, transmigrated PMNs on the lower side of the filter were imaged (Olympus IX81 or Zeiss Axio Observer Z1) and quantified with ImageJ. The screen to identify potential transmembrane proteins that mediate trans-endothelial migration of THP-1 cells was performed in a 96-well format. The ratio of transmigration for each condition was defined as transmigration of THP-1 cells after transfection of HUVECs with an individual siRNA pool divided by the transmigration after transfection with control siRNA. For transmigration assay under flow, $1.5 \times 10^4$ HUVECs were cultured per channel in a fibronectin-coated ibidi μ-Slide I using a parallel-plate flow chamber and stimulated with 10 ng/ml recombinant TNFα (PeproTech) 16 hours prior to the assay. The flow chamber was perfused with HBSS at a constant shear flow (1.2 dyne/cm²) using a computer controlled air pressure pump (ibidi) for 15 min. PMNs were subsequently injected into the perfusion medium and the transmigration process was recorded for 30 min at 0.5 frames/s using a IX81 (Olympus) microscope at 37°C in the presence of 5% CO₂. Percentage of rolling, adhering, crawling and transmigrated PMNs were manually quantified using ImageJ as described previously. Rolling cells were defined as those that move more than 1 cell diameter within 10 s, while adhering cells were those that moved less than 1 cell diameter within 5 s. Cells transmigrating the endothelial monolayer were directly visualized and crawling was defined as the period between adhesion and transmigration.

**Transendothelial electrical resistance measurement**

Endothelial barrier function was assessed in real-time by continuously recording change in trans-endothelial electrical resistance using the ECIS ZTheta system (Applied BioPhysics) as described previously. In brief, 40,000 HUVECs were seeded
per well of a 96W1E + PET electrode plate coated with 1% gelatin (Applied BioPhysics). HUVEC barrier integrity was analyzed after 48 h when cells had formed a confluent monolayer.

**Determination of cell membrane tension**

Membrane tension was determined using the membrane tension probe Flipper-TR® as previously described.\(^1\),\(^10\) Briefly, endothelial cells cultured in ibidi flow chambers or collagen-coated 8-well glass chambers (Nunc) were stimulated with TNFα overnight and incubated with 1 μM of the membrane tension probe Flipper-TR® (Tebu-bio, Cat. No. SC020) for 30 min at 37 °C. Cells were then washed 3 times with HBSS and subjected to flow (1.2 dynes/cm\(^2\)) alone, PMNs alone, or flow together with PMNs, anti-ICAM-1 beads or ICAM-1-clustering antibodies (see below) and imaged with a Leica-SP8 FLIM microscope. Excitation was performed using a pulsed 488 nm laser (Laser kit WLL2+pulse picker, Leica Microsystems) operating at 80 MHz, and the emission signal was collected from 549 to 651 nm with acousto-optical beam splitter (AOBS) using a gated hybrid (HyD SMD) detectors and a TimeHarp 300 TCSPC Module and Picosecond Event Timer (PicoQuant). SymPhoTime 64 software (PicoQuant) was then used to fit fluorescence decay data. To extract lifetime information, the photon histograms from membrane regions were fitted with a double exponential, and 2 fluorescence emission decay times (\(\tau_1\) and \(\tau_2\)) are extracted. The longest lifetime with the higher fit amplitude \(\tau_1\) is used to report membrane tension.\(^1\)

In parallel experiments, we analyzed membrane tension using an alternative approach based on the membrane-bound biosensor MSS.\(^2\) Shortly, HUVECs were transfected with a plasmid encoding the 2 tension sensor modules using Genejuice reagent (Merck Chemicals). FRET experiments were carried out using a Leica SP8 STED 3X confocal microscope with 30 mW argon lasers as described.\(^11\) Briefly, MSS-
expressing cells were subjected to a 440 nm picosecond pulsed laser (Laser kit WLL2+pulse picker, Leica Microsystems) to excite the FRET donor, and the emitted photons were collected over 450-480 nm with internal photon counting mode. The FRET ratio was calculated with Leica LAS X FRET software.

**VE-cadherin internalization assay**

The endocytosis of VE-cadherin was assayed as described. HUVECs were grown to confluency on collagen-coated eight-well glass chamber (Nunc) or fibronectin-coated flow chambers (ibidi, µ-slide I). Cells were stimulated with 10 ng/ml TNFα before they were incubated with 150 μM chloroquine (Sigma, Cat. No. C6628). The antibody against VE-cadherin (Becton Dickinson, Cat. No. 555661) was dialyzed into the cell culture medium and incubated with cells for 1h at 4 °C. Free antibody was removed by rinsing cells in cold EGM-2 medium. Cells were switched back to 37°C and were incubated for 15 min with $1 \times 10^4$ freshly isolated PMNs per chamber without or with flow (1.2 dynes/cm$^2$). PMNs were then removed by rinsing of cells three times with PBS. To remove cell surface-bound antibody while retaining the internalized antibody, cells were washed with PBS (pH 2.7) containing 25 mM glycine and 5% BSA for 15 min. Cells were then fixed with 4% paraformaldehyde and processed for permeabilization and immunofluorescence staining with secondary antibodies Alexa Fluor 488–goat anti-mouse (Invitrogen, Cat. No. A28175), and DNA was stained with DAPI (Invitrogen, Cat. No. D1306). Fluorescence signals were detected with a confocal laser-scanning microscope (Leica SP8 or Olympus C2).

**Mice**

All mice were backcrossed onto a C57BL/6N background at least 10 times, and experiments were performed with littermates as controls. Male and female animals at
an age of 8-12 weeks were used unless stated otherwise. Mice were housed under a 12-hour light-dark cycle with free access to food and water and under specific pathogen-free conditions. The generation of inducible endothelium-specific PIEZO1-deficient mice (Tek-CreERT2;Piezo1^{fl/fl} [EC-Piezo1-KO]) was described previously.⁷ To induce recombination, animals were injected on 5 consecutive days with 1 mg/d tamoxifen (Sigma) dissolved in corn oil, and 10-14 days later experiments were started. All animals which served as controls for tamoxifen-induced endothelium-specific knock-out animals either lacked expression of Cre or the floxed allele and were treated with the same amount of tamoxifen under the same conditions. All procedures of animal care and use in this study were approved by the local animal welfare authorities and committees (Regierungspräsidium Darmstadt, Germany and Ethical Committee of Xi’an Jiaotong University, China).

**Intravital microscopy**

Mice were anesthetized with an intraperitoneal injection of 125 mg/kg ketamine hydrochloride (Zoetis) and 12.5 mg/kg xylazine (Bayer). Cremaster muscle was prepared, and intravital microscopy was carried out as previously described.¹³ Postcapillary venules with a diameter of 20 to 40 µm were chosen for recording using an intravital upright microscope (Zeiss Axio Examiner Z.1) with a 20x W.Plan Apochromat 1.0 numerical aperture saline immersion objective (Zeiss). Inflammation was induced 4 hours before the experiment by intrascrotal injection of interleukin-1β (IL-1β).

**In vivo vascular permeability assay**

A modified Miles assay to determine vascular permeability in the skin was performed as described.¹⁴ Mice were anaesthetized by isoflurane and shaved 2 days before the
assay. Animals were then intravenously injected with 150 µl of 1% Evans blue solution, and 15 min later 20 µl of VEGF (100 ng/ml, PreproTech) or histamine (100 µM, Sigma-Aldrich) in PBS was injected intradermally at the shaved back skin of the mice. 20 µl of PBS was injected as control. 30 min later mice were killed by CO₂, perfused with PBS, and the area around the injection site was dissected. The extravascular dye was then extracted by incubation with formamide at 56°C for 2 days. The degree of vascular leakage was determined by measuring spectrophotometrically at 620 nm the ratio of Evans blue light absorption in test samples and control samples.

Ear dermatitis induced by croton oil

The method of croton oil-induced dermatitis was performed as previously described. Briefly, wild-type or EC-Piezo1-KO mice were treated on the inner surface of the right ear with croton oil (Sigma-Aldrich, 2% v/v in a 4:1 mixture of acetone and olive oil). The left ear was treated with vehicle solution as control. Mice were killed 6 h later, and both ears were harvested and fixed overnight in 4% paraformaldehyde at 4°C in PBS. Ears were then permeabilized with 0.5% Triton X-100, 5% BSA in PBS and incubated in blocking solution (0.5% Triton X-100, 5% BSA in PBS) for 24 h at 4°C. Thereafter samples were incubated with anti-PECAM-1 (BD, clone MEC 13.3, Cat. No. 550274), anti-MRP-14 (R&D Systems, Cat. No. AF2065), or anti-collagen IV (Bio-Rad, Cat. No. 2150-1470) overnight at 4°C. Alexa Fluor 488 donkey anti-goat, Alexa Fluor 594 goat anti-rabbit and Alexa Fluor 647 chicken anti-rat (Thermo Fisher Scientific, Cat. No. A11055, A11037 and A21472, respectively) secondary antibodies were used. Tissue was mounted in fluorescent mounting medium (Polysciences, Cat. No. 18606-5), Z-stack projection were acquired using a Leica SP5 or SP8 confocal microscope and image analysis was performed with Imaris (Bitplane) and ImageJ.
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