SUPPLEMENTARY APPENDIX

Vitronectin stabilizes intravascular adhesion of neutrophils by coordinating β2 integrin clustering

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Animals

Male C57BL/6J mice were purchased from Charles River (Sulzfeld, Germany). Male $VN^{-/-}$ mice, $VN^{+/-}$ mice, and PAI-$1^{-/-}$ were generated as described previously and backcrossed to the C57BL/6J background for 6-10 generations (1, 2). All experiments were performed using mice at the age of 10-12 weeks. Animals were housed under conventional conditions with free access to food and water. The experiments were performed according to German legislation for the protection of animals and approved by the local government authorities.

Reagents

Recombinant mouse CCL2/MCP-1 or CXCL1/KC (0.45 µg in 600 µl PBS i.p. or 0.3 µg in 400 µl PBS i.s.; R&D Systems; Minneapolis, MI) was used to induce leukocyte recruitment. In selected experiments, mouse PAI-1 (CPAI; 50 µg in 100 µl PBS; Molecular Innovations, Novi, MI) and the PAI-1 mutant proteins PAI-QR or PAI-RR (50 µg in 100 µl PBS; Molecular Innovations) were applied i.v. to PAI-$1^{-/-}$ mice. Activation of mouse neutrophils was measured upon exposure to mouse VN, PAI-1, uPA, VN-PAI-1, or VN-uPA (Molecular Innovations). An anti-GPIbα (CD42b) mAb (clone Xia.B2; 50 µg i.v.; 24 h and 6 h prior to induction of inflammation; emfret Analytics, Eibelstadt, Germany) was used for the depletion of platelets. Leukocyte responses were analyzed upon administration of blocking mAbs: anti-CD11a mAbs (clone M17/4; 50 µg in 100 µl PBS i. a.; Biolegend, San Diego, CA, USA), anti-CD11b mAb (clone M1/70; 50 µg in 100 µl PBS i. a.; Biolegend), anti-CD54 mAb (clone YN1/1.7.4; 50 µg in 100 µl PBS i. a.; Biolegend), anti-CD51, anti-CD61/41, Neutrophils were labeled with an anti-Ly6G PE mAb (clone 1A8; 5 µg in 100 µl PBS i. a.; BD Biosciences), platelets were visualized with X-649 (DyLight 649 labelled immunoglobulin derivate; 5 µg in 100 µl PBS i. a.;

emfret Analytics). Endothelial junctions were visualized with a PE-labelled anti-CD31 mAb (clone 390; 10 µg in 100 µl PBS i. a; eBiosciences). mAb24 (detection of high-affinity conformation of β2 integrins; mouse anti-human, monoclonal; abcam) or KIM127 (detection of extended conformation of β2 integrins; mouse anti-human, monoclonal; gift from M. Sperandio) were used to detect specific conformation states of β2 integrins.

VN-PAI-1 heteromers

VN and PAI-1 were mixed at a ratio of 1:2 and heteromers were formed at room temperature for 60 min. Complex formation between VN and PAI-1 was tested in a sandwich ELISA. To this end, the protein mixture was added at increasing concentration (0-750 ng/ml) to anti-PAI-1-, anti-uPA-, or BSA-coated wells (all 2 µg/ml). After washing the wells, the binding of VN-PAI-1 was determined with biotinylated anti-VN, before plates were developed. VN-PAI-1 complexes were detected in a sensitive manner whereas the control antibody showed no non-specific binding.

As further quality control, PAI-1 binding to immobilized VN was tested in comparison to binding to fibronectin (FN) or BSA. VN, FN, or BSA (all 2 µg/ml) was coated in wells and increasing concentration of PAI-1 (0-500 ng/ml) was added. After washing the wells, the binding of PAI-1 was determined with biotinylated anti-PAI-1 antibody. Plates were developed with streptavidin peroxidase and TMB substrate. Absorbance was measured at 450 nm. Binding to BSA-coated wells was subtracted as non-specific binding.

VN-PAI-1 complexes were also measured in citrate mouse plasma after treatment of mice with vehicle or LPS (Sigma Aldrich, 1 mg i.p., 6 h). On anti-PAI-1-coated plates, mouse plasma was added in a dilution of 1:100 and complex formation was measured with a biotinylated anti-VN antibody.

Peritoneal leukocyte trafficking assay

After 6h of i.p. stimulation with CCL2/MCP-1 or CXCL1/KC, mice were sacrificed and their peritoneal cavity was washed with 10 ml of ice-cold PBS. The total number of leukocytes recovered from the peritoneal lavage fluid was analyzed by using a Coulter ACT counter (Coulter Corp.). Samples were then labeled with anti-CD45 APC-Cy7 mAb (clone 30-F11; BD Bioscience), anti-CD11b FITC mAb (clone M1/70; eBioscience, San Diego, CA, USA), anti-Gr-1 PE mAb (clone RB6-8C5; eBioscience), anti-CD115 APC mAb (clone AFS98; eBioscience), and anti-F4/80 eFluor450 mAb (clone BM8; eBioscience) for 30 minutes on ice. Erythrocytes were lysed with lysing solution (1:10; BD FACS lysing solution; BD Bioscience). After two washing steps, leukocytes were resuspended in 250 µl PBS.

Using flow cytometry (Gallios; Beckman Coulter Inc, Brea, CA, USA), myeloid leukocytes were detected by expression of CD45 and Mac-1/CD11b as well as by the absence of F4/80. Thereof, neutrophils were identified by high expression of Gr-1 and low expression of CD115, classical monocytes by high expression of Gr-1 and CD115, and non-classical monocytes by high expression of CD115 as well as by low expression of Gr-1 (see gating strategy in **Fig. 1B**).

In vivo microscopy on the cremaster muscle

- *Surgical procedure*

The surgical preparation of the cremaster muscle was performed as originally described by Baez with minor modifications (3). Mice were anesthetized using a ketamine/xylazine mixture (100mg/kg ketamine and 10mg/kg xylazine), administrated by i.p. injection. The left femoral artery was cannulated in a retrograde manner for administration of microspheres and antibodies. The right cremaster muscle was exposed through a ventral incision of the scrotum. The muscle was opened ventrally in a relatively avascular zone, using careful electrocautery to stop any bleeding, and spread over the pedestal of a custom-made microscopy stage. Epididymis and testicle were detached

from the cremaster muscle and placed into the abdominal cavity. Throughout the procedure as well as after surgical preparation during *in vivo* microscopy, the muscle was superfused with warm buffered saline.

- *Ischemia-reperfusion*

Ischemia of the cremaster muscle was induced by clamping all supplying vessels at the basis of the cremaster muscle using a vascular clamp (Martin, Tuttlingen, Germany). Stagnancy of blood flow was then verified by *in vivo* microscopy (see below). After 30 min of ischemia, the vascular clamp was removed, and reperfusion was restored for 130 min.

- *Experimental protocols*

In a first set of experiments, three postcapillary vessel segments with a vessel diameter of 20-35 µm were randomly chosen in a central area of the spread-out cremaster muscle among those that were at least 150µm away from neighboring postcapillary venules and did not branch over a distance of at least 150µm. *In vivo* microscopy measurements of leukocyte intravascular rolling, adherence, and as well as transmigration were performed 6h after i.s. injection of PBS or i.s. stimulation with CCL2 or CXCL1 or undergoing ischemia-reperfusion (I/R; 30 min/60 and 120 min). After *in vivo* microscopy, blood samples were collected by cardiac puncture for the determination of systemic leukocyte counts using a Coulter ACT Counter (Coulter Corp., Miami, FL, USA). Anesthetized animals were then killed by bleeding to death.

- *In vivo microscopy*

The setup for *in vivo* microscopy was centered around an AxioTech-Vario 100 Microscope (Zeiss MicroImaging GmbH, Goettingen, Germany), equipped with a Colibiri

LED light source (Zeiss MicroImaging GmbH) for fluorescence epi-illumination microscopy as described previously. Light was directed onto the specimen via filter set 62 HE (Zeiss MicroImaging GmbH) fitted with dichroic and emission filters [TFT 495 + 610 (HE); TBP 527 + LP615 (HE)]. Microscopy images were obtained with an AxioCam Hsm digital camera using a 20x water immersion lens (0.5 NA, Zeiss MicroImaging Gmbh). The images were processed with AxioVision 4.6 software (Zeiss MicroImaging GmbH).

- *Quantification of leukocyte kinetics and microhemodynamic parameters*

In vivo microscopy records were analyzed offline in a blinded manner using the imaging software ImageJ (National Institutes of Health, Bethesda, MD) as described previously (4). Briefly, rolling leukocytes were defined as those moving slower than the associated blood flow and quantified for 60s per venule. Firmly adherent cells were determined as those resting in the associated blood flow for 30s and related to the luminal surface per 100µm vessel length. Transmigrated cells were counted in regions of interest (ROI), covering 75µm on both sides of a vessel over 100µm vessel length. To exclude a potential selection bias in determining intravascular adhesion times of leukocytes, the individual adhesion time of each single leukocyte being adherent in the field of view during data analysis at a given time point was measured up to 60 s. By measuring the distance between several images of one fluorescent bead under stroboscopic illumination, centerline blood flow velocity was determined. From measured vessel diameters and centerline blood flow velocity, apparent wall shear rates were calculated, assuming a parabolic flow velocity profile over the vessel cross-section.

- *Cell transfer experiments*

Leukocytes were isolated from donor mice by flushing the femur and tibia bones with PBS. Cells were then sieved and counted, resuspended in PBS containing BSA (0.25%),

and incubated with calcein-AM (10 µmol/L final concentration at 37°C for 30min). After 2 washes, cells were injected into recipient mice via the right jugular vein (10^7 cells/mouse). 120 minutes before the surgical preparation oft he cremaster muscle and induction of I/R (30/120 min). Fluorescent cells were counted in 175 high power fields per animal. Results are shown as the number of accumulated calcein-labeled cells/high power field.

Experimental groups

Animals were assigned randomly to the following groups: C57BL/6J mice, $VN^{-/-}$ mice, and $VN^{+/-}$ mice undergoing 6h of i.p. stimulation with CXCL1, CCL2, or PBS as control (n=4 per group). C57BL/6 mice, $VN^{+/-}$ mice, and $VN^{-/-}$ mice undergoing 6h of i.s. stimulation with CXCL1 or CCL2 (n=4 per group). C57BL/6J mice and $VN^{-/-}$ mice undergoing 30 min of cremasteric ischemia and 120 min of reperfusion (n=4 per group). C57BL/6J mice receiving platelet depleting mAb or isotype control Ab prior to cremasteric I/R injury (30/120 min; n=4 per group). C57BL/6J mice undergoing cremasteric I/R (30/120min) receiving i.a. anti-LFA-1/CD11a, anti-Mac-1/CD11b, anti-ICAM-1/CD54, anti-CD51, anti-CD41/61, or isotype control antibodies (n=4 per group). C57BL/6J mice and $PAI\text{-}1^{-/-}$ mice undergoing 30 min of cremasteric ischemia and 120 min of reperfusion and receiving i.a. CPAI, PAI-RR, or PAI-QR (n=4 per group). C57BL/6J or $VN^{-/-}$ mice undergoing 30 min of cremasteric ischemia and 120 min of reperfusion and receiving i.a. VN-PAI-1 or vehicle (n=4 per group).

Confocal microscopy

For the analysis of VN and PAI-1 deposition on endothelial cells of postcapillary venules, excised mouse cremaster muscles were fixed in 2 % paraformaldehyde. Tissues were then blocked and permeabilized in PBS, supplemented with 10 % goat serum (Sigma Aldrich) and 0.5 % Triton X-100 (Sigma Aldrich). After incubation at 4° C for 12 hours with primary mAb

directed against PECAM-1/CD31 (Invitrogen, Carlsbad, CA), VN, PAI-1 (Abcam), or Ly-6G (Invitrogen), tissues were incubated for 180 min at room temperature with Alexa Fluor 647-, Alexa Fluor 488–linked, or Alexa Fluor 546–linked secondary antibodies. Immunostained tissues were mounted in PermaFluor (Beckman Coulter, Fullerton, CA) on glass slides. Confocal z-stacks typically covering 30 µm (z-spacing 0.5 µm) were acquired using a Leica SP5 confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany) with an oil-immersion lens (Leica; 63x; NA 1.40).

Flow cytometry

To analyze the effect of VN, PAI-1, uPA, VN-PAI-1, and VN-uPA on the expression profile of CD11b/Mac-1 or CD11a/LFA-1 on mouse neutrophils, anticoagulated whole blood samples from C57BL/6J mice were incubated (30 min; 37 °C) with mouse VN, PAI-1, uPA, VN-PAI-1, and VN-uPA or PBS as negative control. After washing, cells were incubated with primary antibodies directed against CD45, Mac-1/CD11b, Gr-1, and CD115 on ice. Isotype-matched controls were used in all experiments. After lysis of erythrocytes, stained cells were analyzed on a flow cytometer (Gallios, Beckmann Coulter). Approximately 20,000 gated events were collected in each analysis.

For the analysis of integrin activation, mouse peripheral blood cells were isolated from male C57BL/6J mice, heparinized and suspended in Hanks Balanced Salt Solution containing 1mM CaCl2 and $MgCl_2$ (Life Technologies, Carlsbad, USA). Cells were exposed to VN, PAI-1, uPA, VN-PAI-1, VN-uPA, or PBS as negative control, in the presence of ICAM-1/CD54-Fc (10 µg/ml, R&D Systems) and PE-conjugated anti–human IgG1 (Fc-specific, Southern Biotechnology) for 5min at 37°C. Additionally, isolated blood cells were treated with RAP (Molecular Innovations), anti-LRP-1 ab (BioMac, Leipzig, Germany), SB203580 (25 µM; Sigma Aldrich), SP600125 (5 µM; Sigma Aldrich), FR180204 (15 µM; Sigma Aldrich), or vehicle/control IgG prior to stimulation with VN-PAI-1. After washing, cells were labeled with antibodies directed against CD45,

Mac-1/CD11b, CD115, and GR1. Binding of ICAM-1/CD54 was measured by a flow cytometer (Gallios, Beckmann Coulter). The results are analyzed with FlowJo Software (Treestar).

In separate experiments, anticoagulated whole blood from healthy human donors was stimulated incubate with human VN-PAI-1 (Molecular Innovations) or PBS. Neutrophils were identified by size and granularity. Binding of mAb24 (detection of high-affinity conformation of β2 integrins; mouse anti-human, monoclonal; abcam) or KIM127 (detection of extended conformation of β2 integrins; mouse antihuman, monoclonal; gift from M. Sperandio) was measured by flow cytometry (see above).

Autoperfused flow chamber assay

To study neutrophil rolling and adhesion in a reductionist system, we used a previously described *ex vivo* flow chamber system (5). Briefly, glass capillaries (Rect. Boro Capillaries 0.04x0.40mm cross section, VitroCom) were coated overnight with different combinations of rmE-selectin (CD62E Fc chimera, R&D Systems), rmICAM-1 (ICAM-1 Fc chimera, R&D Systems), rmCXCL1 (Peprotec, London, UK) and/or rmVN-PAI-1 heteromers (Molecular Innovations) and blocked with 5% casein (Sigma-Aldrich) for 2 h. Flow chambers were then perfused by whole blood through a carotid artery catheter which had been placed into anesthetized WT control mice. An appropriate field of view along the flow chamber was recorded for 10min using an Olympus BX51WI microscope with a CCD camera (model CF8/1, Kappa) and a water immersion objective (x40/0.8 NA, Olympus) and analyzed for rolling and adhesion using Fiji software (6).

In vitro neutrophil detachment assay

Adhesion strengthening of isolated mouse bone marrow derived neutrophils was analyzed in a dynamic in vitro neutrophil detachment assay using Ibidi-Slide IV 0.1 flow chambers (Ibidi, Munich, Germany) coated with rmE-selectin (CD62E, R&D), rmICAM-1

(ICAM1/CD54, R&D), CXCL1/KC (Peprotech) and VN-PAI-1 (Molecular Innovations). Neutrophils were initially placed into flow chambers for 5min for attachment on the coated chamber surface before shear stress was increased gradually (30s steps) to 40dyn/cm^2 using a high-precision syringe pump (KD Scientific Holliston).

β_2 integrin clustering and actin colocalization under increasing shear stress

To study integrin cluster formation under flow conditions, the cell surface expression of lymphocyte function-associated antigen-1 (LFA-1, $\alpha_L\beta_2$) in neutrophils was analyzed following the adhesion-strengthening assay described above. Before imaging, the cells were stained with a PE-labeled anti-CD11a antibody (1µg/ml, 2D7, BD Pharmingen) for 15min and with SiR-actin (100nM. Spirochrome) overnight. Images were acquired with an upright spinning disk confocal microscope (Examiner, Zeiss) using a 20x/1.0 water immersion objective. The microscope was equipped with a confocal scanner unit CSUX1 (Yokogawa Electric Corporation). LFA-1 expression was assessed by fluorescence detection upon excitation with a 561-nm laser and an exposure time of 100ms. Actin expression was assessed by fluorescence detection upon excitation with a 640-nm laser and an exposure time of 100 ms. Images for a single z-stack were recorded every 1s using an EMCCD camera (Evolve 512, Photometrics). LFA-1 sum intensities per single cell were measured in the area colocalizing with actin using Fiji's implemented plugin colocalization threshold (6). LFA-1 clustering was defined by a segmentation mask using an intensity threshold of a minimum of 95% of the maximum clustering. Acquisition and analysis were performed using Slidebook (6.0.8, 3i), Fiji (NIH) and Flowjo (10.1 Flowjo LLC) software.

Statistics

Data analysis was performed with a statistical software package (SigmaStat for Windows; Jandel Scientific). Rank Sum test (two groups) or ANOVA-on-ranks followed

by the Dunnett test (> two groups) were used for the estimation of stochastic probability in intergroup comparisons. Mean values and SEM are given. *p*-Values < .05 were considered significant.

Supplemental Tables

Supplemental table 1

mouse	stimulus	treatment	SLC [x 10^3/µl]	inner vessel diameter [µm]	v_{mean} [mm/s]	shear rate [1/s]
WT	I/R	-	2.4 ± 0.5	28.6 ± 0.7	1.0 ± 0.1	1238.2 ± 126.2
$VN^{-/-}$	I/R	-	1.8 ± 0.1	28.3 ± 0.7	0.9 ± 0.3	1197.9 ± 141.8
WT	CXCL1	-	1.8 ± 0.9	29.2 ± 0.3	1.0 ± 0.2	1350.0 ± 135.2
$VN^{+/-}$	CXCL1	-	2.4 ± 0.6	25.3 ± 1.2	1.3 ± 0.2	1264.2 ± 145.7
$VN^{-/-}$	CXCL1	-	2.3 ± 0.2	26.9 ± 0.7	1.2 ± 0.1	1309.9 ± 150.2
WT	CCL2	-	2.5 ± 1.3	30.1 ± 0.6	0.9 ± 0.1	1272.5 ± 121.2
$VN^{+/}$-	CCL2	-	2.6 ± 0.8	28.4 ± 0.3	1.0 ± 0.2	1309.6 ± 145.7
$VN^{-/-}$	CCL2	-	2.0 ± 0.9	25.9 ± 0.7	1.4 ± 0.4	1149.8 ± 118.7
WT	I/R	WT BM cells	2.9 ± 0.4	24.8 ± 1.5	1.1 ± 0.1	1222.4 ± 149.2
$VN^{-/-}$	I/R	WT BM cells	2.7 ± 0.6	29.9 ± 0.7	0.9 ± 0.1	1371.6 ± 81.3
WT	I/R	$VN^{-/-}$ BM cells	3.0 ± 1.0	28.8 ± 2.0	0.9 ± 0.1	1315.2 ± 102.2
WT	I/R	isotype control	2.7 ± 0.9	29.8 ± 1.2	1.5 ± 0.4	1355.6 ± 140.0
WT	I/R	anti-CD42 mAb	2.4 ± 0.8	26.9 ± 0.9	1.2 ± 0.1	1324.2 ± 89.7
WT	I/R	isotype control	2.8 ± 0.6	31.3 ± 1.5	0.9 ± 0.1	1188.2 ± 149.7
WT	I/R	anti-CD11a mAb	1.8 ± 0.5	25.4 ± 1.2	0.9 ± 0.1	1302.6 ± 108.2
WT	I/R	anti-CD11b mAb	2.3 ± 0.2	26.2 ± 0.7	1.0 ± 0.2	1311.6 ± 84.3
WT	I/R	anti-CD54 mAb	2.9 ± 1.3	25.0 ± 0.7	1.1 ± 0.2	1208.8 ± 55.9
PAI-$1^{-/-}$	I/R	vehicle	2.7 ± 0.9	29.2 ± 0.3	1.1 ± 0.1	1178.8 ± 89.2
PAI-$1^{-/-}$	I/R	PAI-RR	1.8 ± 0.9	27.3 ± 1.2	0.9 ± 0.1	1189.1 ± 105.7
PAI-$1^{-/-}$	I/R	PAI-QR	2.8 ± 0.4	26.9 ± 0.3	1.3 ± 0.2	1212.8 ± 63.7
PAI-$1^{-/-}$	I/R	CPAI	2.0 ± 0.3	30.1 ± 0.7	1.1 ± 0.1	1202.5 ± 89.2
WT	I/R	-	2.6 ± 0.5	29.1 ± 0.6	1.0 ± 0.1	1322.1 ± 143.5
$VN^{-/-}$	I/R	vehicle	2.8 ± 1.2	27.5 ± 0.7	0.9 ± 0.1	1289.7 ± 121.3
$VN^{-/-}$	I/R	VN-PAI-1	2.7 ± 0.8	28.1 ± 0.4	0.9 ± 0.1	1260.5 ± 137.1

Table S1. Systemic leukocyte counts and microhemodynamic parameters. Systemic leukocyte counts and microhemodynamic parameters were measured as detailed in *Methods*. Quantitative data are shown (mean±SEM for n=4 per group).

Supplemental figures

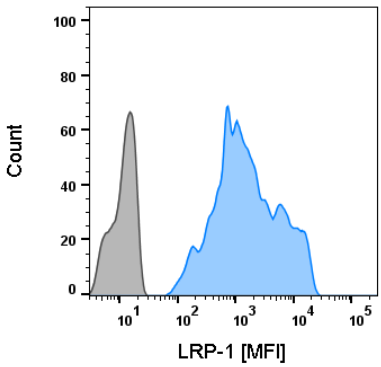

Figure S1. Expression of LRP-1 on mouse neutrophils. Heterogenous surface expression of LRP-1 in neutrophils isolated from the peripheral blood of WT mice as assessed by immunostaining and multi-channel flow cytometry as detailed in *Methods*. A representative histogram plot is shown (anti-LRP-1 mAb in blue, isotype control in grey).

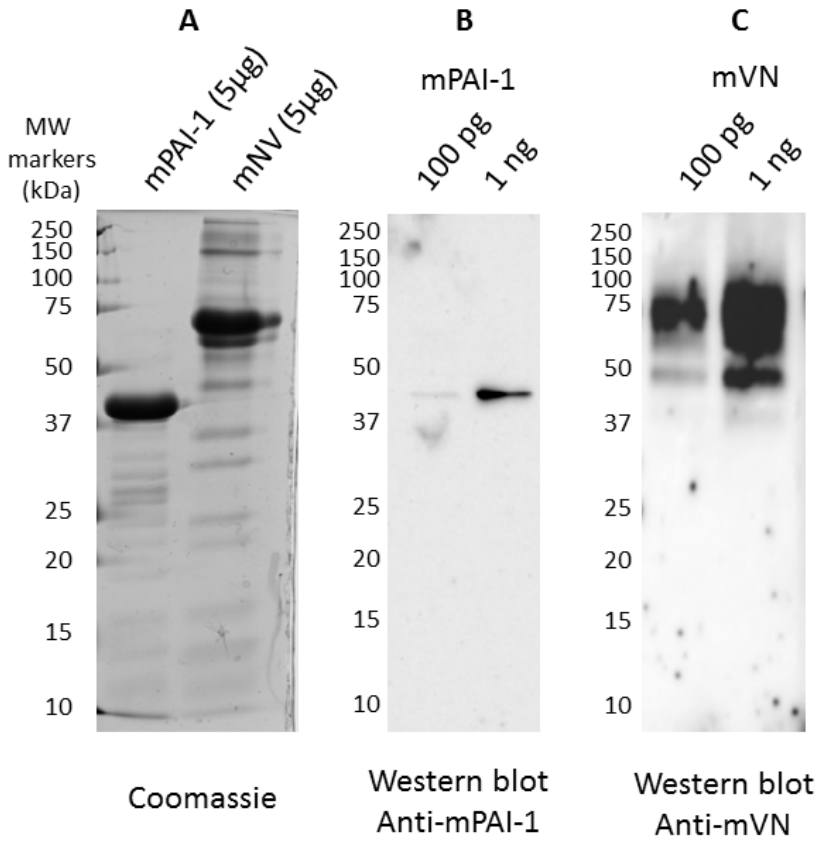

Figure S2. Analysis of mouse vitronectin (mVN) and mouse plasminogen activator inhibitor-1 (mPAI-1) by Coomassie staining and Western blotting. (**A**) 5 μg of each protein was run on SDS-PAGE under reducing conditions (10% v/v β-mercapto-ethanol) and the gel was stained with Coomassie blue. Mouse multimeric VN (68 kDa) was isolated by the vendor from mouse plasma by urea denaturation and heparin affinity chromatography and mPAI-1 was recombinant (43 kDa). (**B**) 100 pg and 1 ng of mPAI-1 was run under reducing conditions and Western blotting was performed with a rabbit polyclonal anti-mPAI-1 antibody. (**C**) 100 pg and 1 ng of mVN was run under reducing conditions and Western blotting was performed with a rabbit polyclonal anti-mVN antibody.

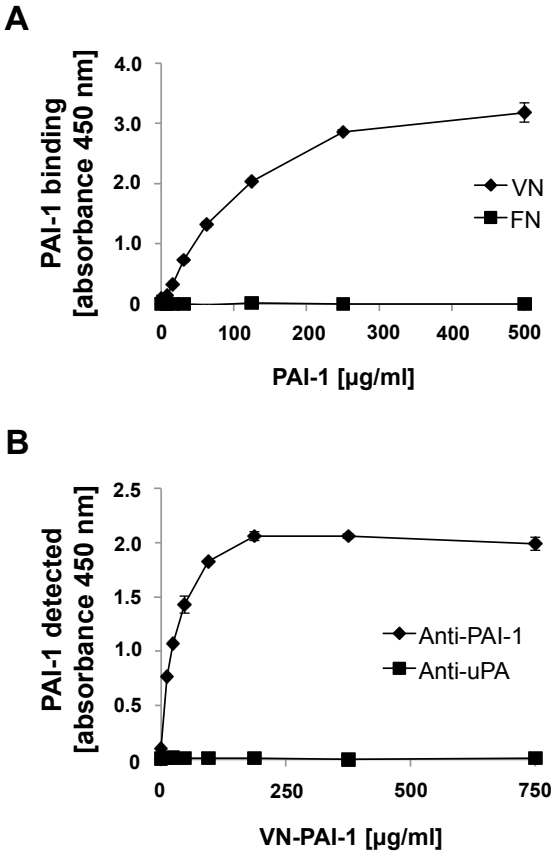

Figure S3. VN-PAI-1 heteromers. Binding of recombinant mouse PAI-1 to mouse vitronectin (VN) or fibronectin (FN; as negative control) was determined *in vitro* by ELISA analysis as detailed in *Methods* (**A**). Formation of mouse VN-PAI-1 heteromers was assessed *in vitro* by sandwich ELISA analysis as detailed in *Methods* (**B**). Quantitative data are shown (mean±SEM for n=3 per group).

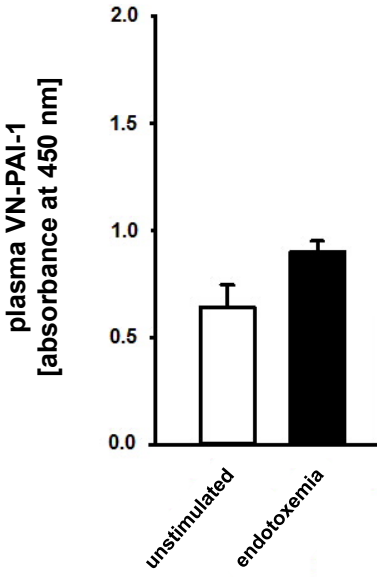

Figure S4. VN-PAI-1 heteromers in the systemic circulation. Plasma levels of VN-PAI-1 heteromers in the peripheral blood of unstimulated WT mice and of WT mice challenged with LPS were assessed by sandwich ELISA analysis as detailed in *Methods*. Quantitative data are shown (mean±SEM for n=5 per group).

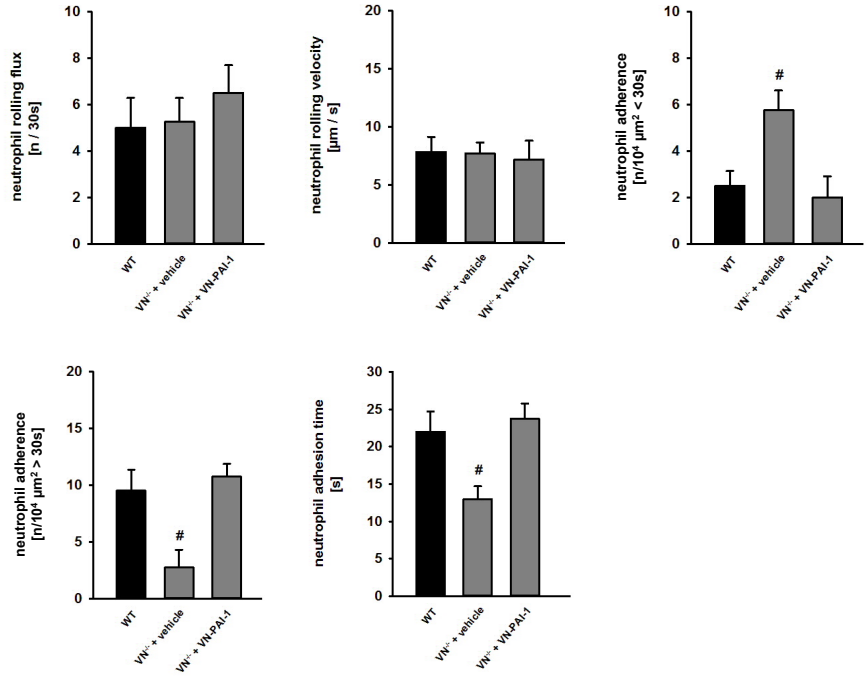

Figure S5. Rescue of neutrophil firm adherence in VN$^{-/-}$ mice by substitution of VN-PAI-1 heteromers. Intravascular rolling, rolling velocity, short adhesion (adherence < 30s), firm adherence (adherence >30s), and average adhesion time of neutrophils were assessed by multi-channel *in vivo* microscopy in the cremaster muscle of WT mice and VN$^{-/-}$ mice undergoing 30 min of ischemia and 120 min of reperfusion as well as receiving mouse VN-PAI-1 or vehicle as detailed in *Methods*. Quantitative data are shown (mean±SEM for n=4 per group; #p<0.05 vs WT).

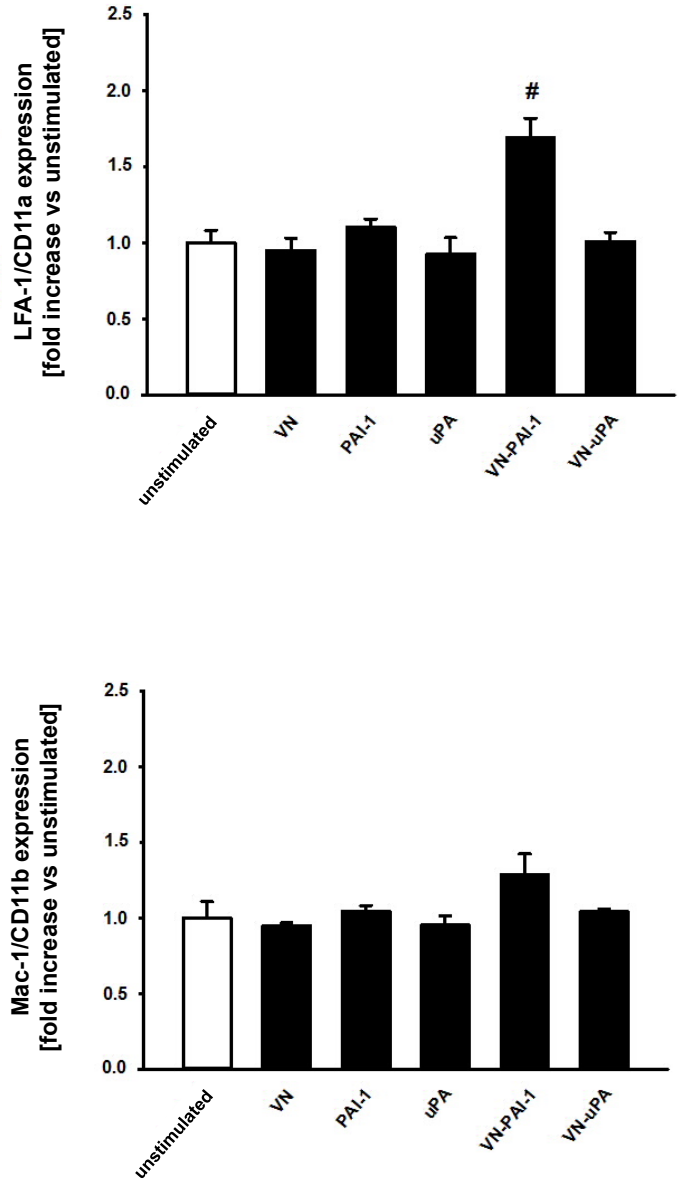

Figure S6. Effect of VN-PAI-1 heteromers on expression of β2 integrins on neutrophils. Surface expression of the β2 integrins LFA-1/CD11a and Mac-1/CD11b on neutrophils isolated from the peripheral blood of WT mice was assessed by multi-channel flow cytometry upon exposure to mouse VN, PAI-1, uPA, VN-PAI-1, VN-uPA protein, or vehicle as detailed in *Methods*. Quantitative data are shown (mean±SEM for n=4-6 per group).

Supplemental videos

Supplemental video 1. Leukocyte-endothelial cell interactions in WT mice. Employing *in vivo* microscopy, interactions of leukocytes (neutrophils: red) and microvascular endothelial cells were visualized in a postcapillary venule (inner diameter: approximately 25 µm) of the cremaster muscle of a C57BL/6 mouse 6 h after intrascrotal stimulation with recombinant mouse CXCL1/KC.

Supplemental video 2. Leukocyte-endothelial cell interactions in VN-deficient mice. Employing multi-channel *in vivo* microscopy, interactions of leukocytes (neutrophils: red) and microvascular endothelial cells were visualized in a postcapillary venule (inner diameter: approximately 25 µm) of the cremaster muscle of a $VN^{-/-}$ mouse 6 h after intrascrotal stimulation with recombinant mouse CXCL1/KC.

Supplemental References

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