Supplemental Methods

FGD5 staining of cremaster wholemounts

For labelling of cremasteric vessels with FGD5 antibodies, FGD5 or control siRNA treated mice were perfused with 1% PFA/PBS for 2 min followed by *in situ* pre-fixation of the cremaster muscle with 4% PFA/PBS for 8 min. The cremaster was prepared, post-fixed in 1% PFA/PBS, blocked and permeabilized in 5% donkey serum, 0.2% BSA, 0.3% Triton-X100, 0.1% NaN_3/PBS for 4-5 h followed by antibody labelling. During primary antibody incubation, blocking reagents were present while secondary antibody incubation was performed without any blocking reagent.

Isolation of platelets

Angpt1$^{lox/lox}$ or *Angpt1*^{PltKO} mice were anaesthetized by isoflurane inhalation and maintained under anesthesia using an isoflurane-vaporizer (Harvard Apparatus). Blood was collected from the *vena cava* into a syringe containing ACD anticoagulant (23 mM citric acid monohydrate, 45 mM trisodium citrate anhydrate, 63 mM D-glucose). Blood and ACD buffer were mixed, collected in a tube containing BSG-citrate buffer (117 mM NaCl, 16 mM trisodium citrate anhydrate, 11 mM D-glucose, 1.6 mM KH_2PO_4, 4.6 mM Na_2HPO_4) and submitted to centrifugation at 200 x g for 3 min at RT. Plasma and buffy coat were collected and centrifugation was repeated. To prevent activation of platelets, Prostaglandin E_1 (Sigma) was added at a final concentration of 1 µM in modified Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 0.42 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5.5 mM D-glucose), incubated for 5 min at RT and followed by centrifugation at 900 x g for 5 min at RT. The platelet pellet was washed and submitted to cell lysis.

Angpt1 ELISA

Platelet lysates from *Angpt1*$^{lox/lox}$ or *Angpt1*^{PltKO} mice were submitted to the PikoKine Angpt1 ELISA (Bosterbio) according to the manufacturer's instructions. In brief, diluted platelet lysates were incubated on the capture-antibody-coated microplate for 90 min, labelled by biotinylated anti-Angpt1 antibodies and avidin-peroxidase complexes and levels of Angpt1 were determined by reading OD450 nm in a microplate reader (Synergy 2, BioTek).

FITC-dextran leakage in the inflamed cremaster

Mice received i.p. injections of 100 µg anti-Ly6G/Gr-1 antibodies to deplete neutrophils or anti-rat control IgG 24 h before a local inflammation was induced by intrascrotal injection of 50 ng IL-1β (Biomol) for 4 h total. 2 h after IL-1β injection, 40 kDa FITC-dextran was injected i.v. and

allowed to circulate for 2 h before mice were sacrificed and the cremaster was prepared as described in the main manuscript.

Confocal intravital microscopy of cremaster whole mounts

10-14 week-old male LysM-eGFP mice were intrascrotally injected with 50 ng IL-1β. Endothelial cells and platelets were labeled through intravenous injection of Alexa-Fluor-555-coupled anti-PECAM-1 antibodies (30 µg/mouse, 390) and DyLight-649-coupled anti-GPIbβ antibodies (0.1 µg/g body weight, X649), respectively. 1 h after IL-1β stimulation, mice were anaesthetized and the cremaster muscle was exteriorized. Surgical preparation was performed as previously described[1,2]. In each animal, up to two unbranched postcapillary venules with a diameter of 15-25 µm were analyzed. 1-1.5 h videos were taken over a period of 2 h using a confocal microscope (Zeiss LSM880 with Airyscan Fast Mode). Imaris software (Bitplane) was used to evaluate the videos.

Supplemental References

1. Bixel MG, Petri B, Khandoga AG, et al. A CD99-related antigen on endothelial cells mediates neutrophil but not lymphocyte extravasation in vivo. *Blood*. 2007;109(12):5327-5336.

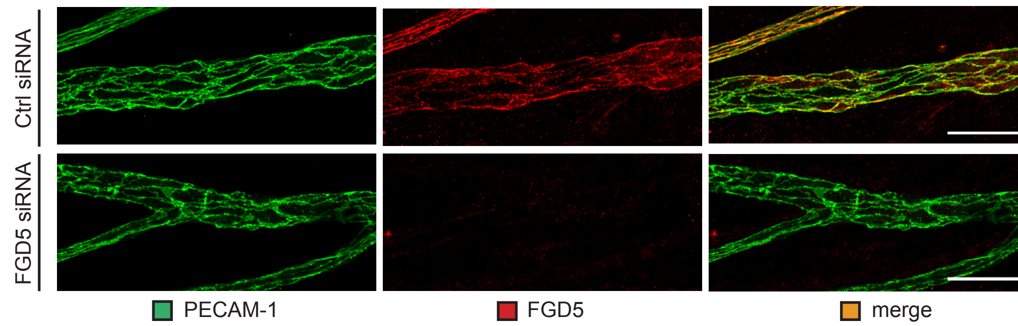
2. Broermann A, Winderlich M, Block H, et al. Dissociation of VE-PTP from VE-cadherin is required for leukocyte extravasation and for VEGF-induced vascular permeability in vivo. *J Exp Med*. 2011;208(12):2393-2401.

Supplemental Data

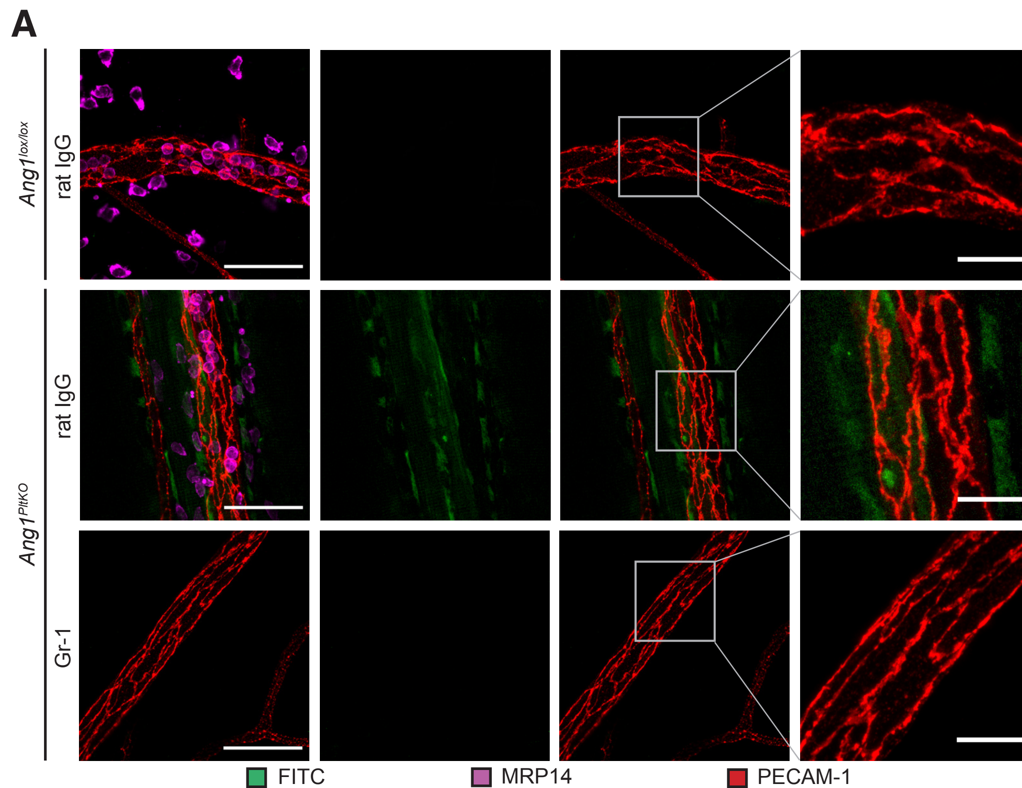
Video 1:

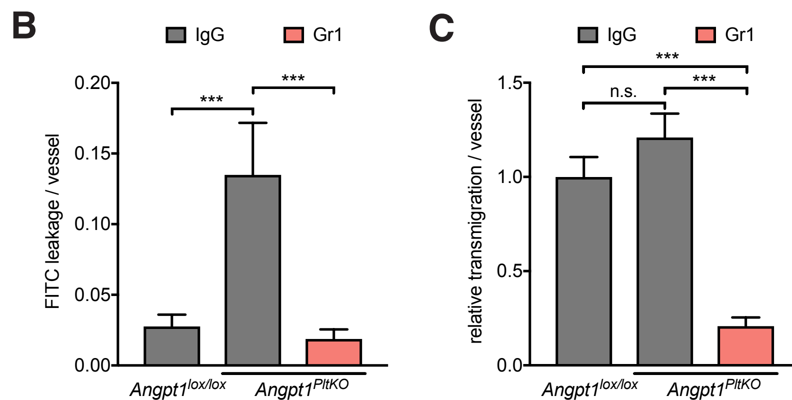
Platelet-rich areas mark preferential sites of neutrophil extravasation. Confocal intravital video microscopy of IL-1β-stimulated cremasteric venules in *LysM-eGFP* mice. Endothelial cells (red) and platelets (white) were labeled via intravenous injection of anti-PECAM-1- and anti-GPIbβ antibodies, respectively. Cremaster muscle was prepared under anesthesia and 1-1.5h videos were recorded.

Open arrowheads mark platelets bound to the endothelium, which remain in the same position for the duration of the video. White arrowheads mark areas of neutrophil transmigration. Importantly, neutrophils only exit at platelet-rich areas (upper endothelial lining) and not in regions devoid of platelets (lower endothelial lining). Timestamp (lower right). Scale bar 10 μm.

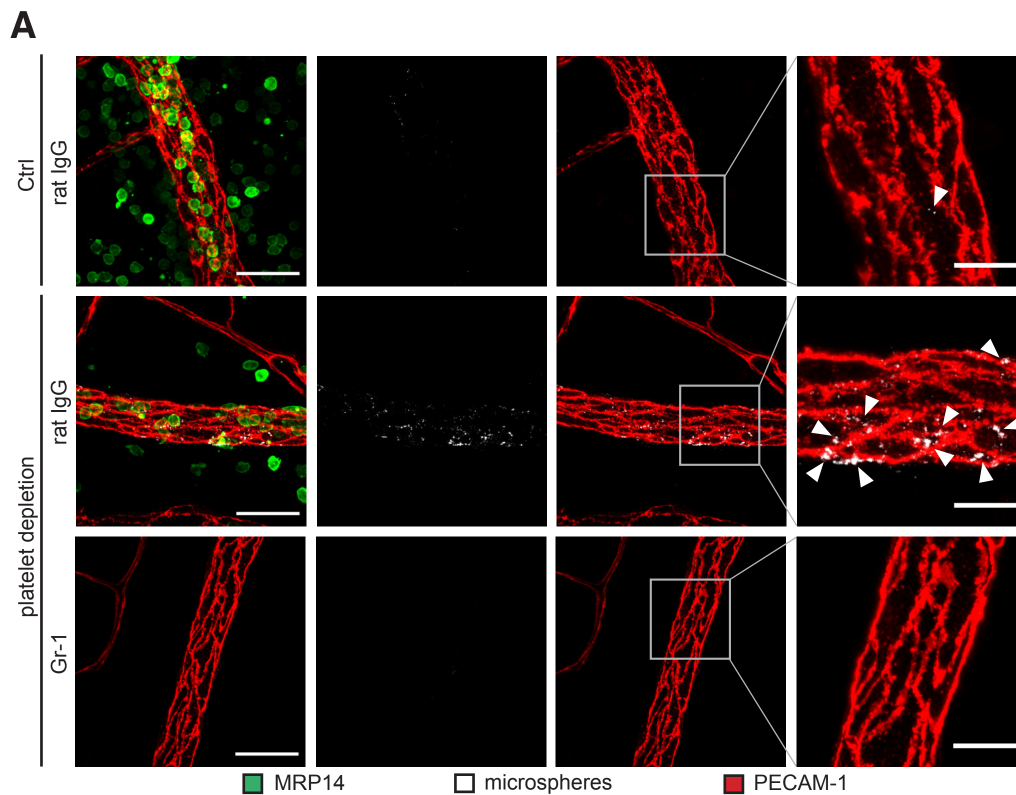


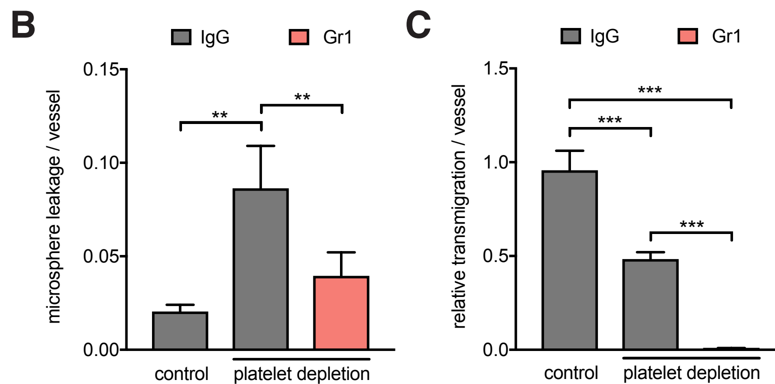
Suppl. Figure 1:
FGD5 siRNA efficiently reduces FGD5 expression in vessels of the cremaster.
C57Bl/6 mice were intrascrotally injected with FGD5 or control siRNA and 48 h later, cremaster muscle wholemounts were prepared and stained for FGD5 and PECAM-1. Scale bar 50 µm. Pictures are representative of three independent experiments.





Suppl. Figure 2:
Platelet-derived Angpt1 prevents dextran leakage during transmigration of neutrophils. *Angpt1*$^{lox/lox}$ or *Angpt1*^{PltKO} mice were i.p. injected with anti-Gr-1 or control IgG antibodies to deplete neutrophils and 24 h later intrascrotally stimulated with IL-1β. After 2 h, 40 kDa FITC-Dextran was i.v. injected and allowed to circulate for 2 h. Then, cremaster wholemounts were prepared and stained for PECAM-1 and MRP14. Scale bars 40 μm or 15 μm in detailed view. FITC-dextran leakage per vessel (B) and transmigrated neutrophils per vessel (C) were quantified. Results are representative of (A) or pooled from (B, C) three independent experiments with a total number of 30 vessels analyzed.





Suppl. Figure 3:
Platelet depletion results in neutrophil-induced leaks during transmigration *in vivo*. (A) C57Bl/6 were injected i.p. with anti-Gr-1 or control IgG antibodies to deplete neutrophils and stimulated intrascrotally with IL-1β 24 h later. After 3 h, they received i.v. injections of anti-GPIbα antibodies (R300) or control IgG antibodies to deplete platelets and, another hour later, fluorescent microspheres were given i.v. for 5 min. Cremaster wholemounts were prepared and stained for PECAM-1 and MRP14. Scale bars 40 μm or 15 μm in detailed view. Microsphere leakage per vessel (B) or transmigration of neutrophils per vessel (C) were quantified. Results are representative of four independent experiments with a total number of 40 vessels analyzed.