

Src family kinase-mediated vesicle trafficking is critical for neutrophil basement membrane penetration

Ina Rohwedder,¹ Angela R.M. Kurz,¹ Monika Pruenster,¹ Roland Immler,¹ Robert Pick,¹ Tanja Eggersmann,¹ Sarah Klapproth,¹ Jennifer L. Johnson,² Sergi Masgrau Alsina,¹ Clifford A. Lowell,³ Attila Mócsai,⁴ Sergio D. Catz² and Markus Sperandio¹

¹Walter-Brendel-Center of Experimental Medicine, Institute of Cardiovascular Physiology and Pathophysiology, Klinikum der Universität, Ludwig-Maximilians-University Munich, Planegg-Martinsried, Germany; ²Department of Molecular Medicine, The Scripps Research Institute, La Jolla, CA, USA; ³Department of Laboratory Medicine, University of California, San Francisco, CA, USA and ⁴Department of Physiology, Semmelweis University School of Medicine, Budapest, Hungary

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Correspondence: *MARKUS SPERANDIO* - markus.sperandio@lmu.de

Supplemental Data

Detailed methods

Intravital microscopy of TNF α –stimulated mouse cremaster muscle venules.

Intravital microscopy of mouse cremaster muscle venules was performed in wildtype and SFK-ko mice. Briefly, mice were treated for 2h by intrascrotal injection of 500ng recombinant murine TNF α (R&D Systems, Minneapolis, USA) and a carotid artery catheter was placed for blood sampling using a ProCyte Dx Hematology Analyzer (IDEXX, Westbrook, USA). Movies from mouse cremaster muscle postcapillary venules ranging from 20-40 μ m in diameter were recorded using a BX51WI microscope equipped with a water immersion objective (\times 40, 0.80 NA, Olympus) and a CCD camera (CF8/1, Kappa). Neutrophil adhesion efficiency (number of adherent cells/mm² divided by the systemic neutrophil count) was calculated on the basis of the recorded movies using Fiji software¹. Transmigrated cells were calculated using dissected, fixed and Giemsa (Merck Millipore, Darmstadt, Germany) stained cremaster muscle whole mounts. Analysis was performed using a Zeiss Axioskop 40 microscope equipped with an oil immersion objective x100, 1.25 NA (Zeiss, Jena, Germany).

Multi-photon microscopy of the mouse cremaster muscle.

Neutrophils from SFK-ko or wildtype mice were isolated and after labeling with CellTracker™ Deep Red Dye (ThermoFisher), 1×10^7 cells were injected via tail vein into Lyz2^{GFP} or SFK-ko x Lyz2^{GFP} respectively. 1h later TNF- α (500 ng/mouse) and a rat anti- CD31 antibody Alexa Fluor 546 were injected into the scrotum of mice. 2h later, the cremaster muscle was prepared for intravital multi-photon microscopy. Multi-photon microscopy was performed at the core facility BioImaging of the Biomedical Center with a Leica SP8 MP microscope, equipped with a pulsed InSight DS+ laser. Excitation of all dyes was with 860 nm. Images were acquired with a 20x1.0 objective. Multi-photon excited images were recorded with external, non-descanned hybrid photo detectors (HyDs). Images (512 \times 512 pixels, step size 5 μ m) were acquired at intervals of 30 seconds. After acquisition, images were processed and analyzed using Imaris 7 (Bitplane).

TNF α –induced neutrophil extravasation into the peritoneal cavity.

Mice were injected i.p. with 0.9% NaCl (unstimulated) or TNF α (2 μ g/mouse) and sacrificed 2 h later. Peritoneal lavage was performed and the number of extravasated neutrophils was evaluated using rat anti-Ly6G antibody (1A8; BioLegend, San Diego, USA), Flow-Count Fluorospheres (Beckman Coulter, Brea, USA) and flow cytometry (Gallios flow cytometer, Beckman Coulter).

In vitro crawling and detachment assays

Glass capillaries (Rect.Boro Capillaries 0.04x0.40mm ID VitroCom, Mountain Lakes, USA) were coated for 3h with a combination of 20µg/ml E-Selectin and 15µg/ml ICAM-1 (Fc chimera, R&D Systems) and 15µg/ml CXCL1. Whole blood was collected from SFK-ko and wildtype mice via the cannulated carotid artery. Neutrophils were allowed to attach for 3min, then chambers were flushed and detachment assays performed over 10min with increasing flow rates (7 – 270 dyne/cm²) every 30s and recorded as time lapse movie using an upright microscope (Zeiss Axioskop2 provided with a 20x water objective, 0.5NA and a Hitachi KP-M1AP camera). Number of attached cells was counted at the end of each step.

LFA1 clustering

Flow chambers were prepared as described above and were perfused with whole blood from SFK-ko and wildtype mice, which had been incubated for 10min with a non-blocking rat anti mouse LFA1 Alexa Fluor 546 antibody (clone 2D7, BioLegend, labeled with a monoclonal antibody labeling kit (Thermo Fisher)). Adherent cells were imaged using a confocal microscope (Leica System SP5, 63x, 1.4 NA oil immersion objective, xyzt-series). Videos were analyzed for LFA-1 signal intensity offline using Fiji software.

Transwell assay

Murine neutrophils were isolated from bone marrow using EasySep™ mouse neutrophil enrichment kit (STEMCELL TECHNOLOGIES, Vancouver, Canada). A transwell assay (3µm pore size) was performed using either complete HBSS buffer or HBSS buffer containing 1, 10 or 100ng CXCL1 (Peprotech, Rocky Hills, USA) as chemoattractant. Isolated neutrophils were applied to the upper compartment and allowed to migrate for 45min at 37°C. Cells were collected from the lower chamber and the number of transmigrated neutrophils was evaluated using rat anti-Ly6G antibody (1A8; BioLegend), Flow-Count Fluorospheres (Beckman Coulter) and flow cytometry

For BM transmigration assays the membrane was coated overnight (ON) with 15µg/ml laminin 1 (Thermo Fisher, Waltham, USA) and afterwards with a combination of 2µg/ml PECAM-1 and 8µg/ml ICAM-1 (both R&D Systems) for 2h at 37°C. Either HBSS buffer alone or HBSS buffer containing 1ng/ml CXCL1 was added to the lower compartment. Isolated neutrophils (2x10⁵ cells per well) were allowed to migrate for 3h at 37°C. Transmigrated cell number was determined as described.

Vesicle mobilization assay

Analysis of vesicle mobilization on PECAM-1, ICAM-1, and CXCL1-coated wells was performed as previously described ². Briefly, slides were coated with either 2% BSA (control; GE Healthcare) or PECAM-1 (2µg/ml; R&D Systems), ICAM-1 (8µg/ml; R&D Systems) and CXCL1 (10µg/ml; PeproTech) overnight at 4°C. Isolated bone marrow neutrophils from wildtype, or SFK-ko mice were incubated on coated slides for 30 min at 37°C. Cells were fixed with 4% PFA, blocked, permeabilized and stained with mouse anti-VLA3 (42/CD49c; BD), rat anti-VLA6 (GoH3/CD49f; BioLegend), rabbit anti-NE (polyclonal; Abcam), mouse anti-Rab27a (Transductions Laboratories R52320), rabbit anti-JFC1 ³ and Munc13-4 ⁴ and respective secondary antibodies (all Molecular Probes/Invitrogen). Cells were imaged by confocal microscopy (Leica SP5 System, ×63, 1.4 NA oil immersion objective). Images were analyzed offline using Fiji software.

In vivo NE activity assay

NE680FAST (4 nmol/mouse; Perkin-Elmer) was injected i.v. and 1 hour later TNFα was applied i.s. to wildtype and SFK-ko mice. After 2 hours, the cremaster muscle was dissected and fixed with 4% paraformaldehyde, permeabilized and blocked with 0.5% Triton X-100/2% BSA in PBS, stained with rat anti-CD31 antibody-Alexa Fluor 488 (MEC13.3; BioLegend). Tissue was embedded on glass slides in PermaFluor (Thermo Scientific) and imaged by confocal microscopy (Leica SP5 system, ×40, 1.4 NA oil objective).

Flow cytometry

Surface expression of CD11a (LFA1, αL -APC, eBioscience), CD11b (MAC-1, αM -Brilliant Violet 570 BioLegend), CXCR2 (CD182-APC R&D Systems), CD44 (CD44-Brilliant-Violet 570, BioLegend), PSGL1 (CD162-PE, Pharmingen) and L-selectin (CD62L-FITC, BioLegend) was compared for whole blood derived Ly6G⁺ (Ly6G-Pacific Blue, BioLegend) neutrophils obtained from SFK-ko and wildtype mice using a Beckman Coulter GalliosTM flow cytometer and analyzed using Kaluza[®] Flow Analysis Software (Beckman Coulter).

MPO quantitative ELISA

Mouse serum from wildtype and SFK-ko was obtained 2h after i.p. injection of 0.9% NaCl (unstimulated) or TNFα (1 µg/mouse). Quantitative ELISA was performed according to the manufacturers' instructions (Hycult Biotech, Uden, Netherlands)

SDS-PAGE and immunoblotting

Bone marrow derived neutrophils were seeded on ICAM-1 coated coverslips for 10min. Cells were stimulated with CXCL1 or PMA (Phorbol-12-myristat-13-acetat) for 20min at 37°C, lysed and proteins were resolved by SDS-polyacrylamide (SDS-PAGE) gels and then electrophoretically transferred from the gels onto PVDF membranes, which were subsequently blocked in LI-COR blocking solution (Lincoln, USA) and incubated with antibodies. The following antibodies were used for detection: p-Paxillin, Paxillin, p-Syk, Syk, p-Cortactin and Cortactin all purchased from CellSignaling (Danvers, USA). IRDye 680RD and IRDye800CW-coupled secondary antibodies were purchased from LI-COR. Western blots were scanned using the Odyssey® CLx Imaging System (LI-COR) and analyzed with Image Studio software.

Statistics

All data were analyzed and plotted using GraphPad Prism 7 software (GraphPad Software Inc). For pairwise comparison of experimental groups, a paired t-test, unpaired t-test, or Mann-Whitney test was performed. For comparison of 3 or more samples, a 1-way ANOVA was used with Dunnett's multiple comparisons test. For multiple comparisons, a 2-way ANOVA was used with either Sidak's multiple comparisons test or Tukey's multiple comparisons test. A *P* value less than 0.05 was considered as statistically significant and indicated with *. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$. n.s. = not significant.

Figure S1

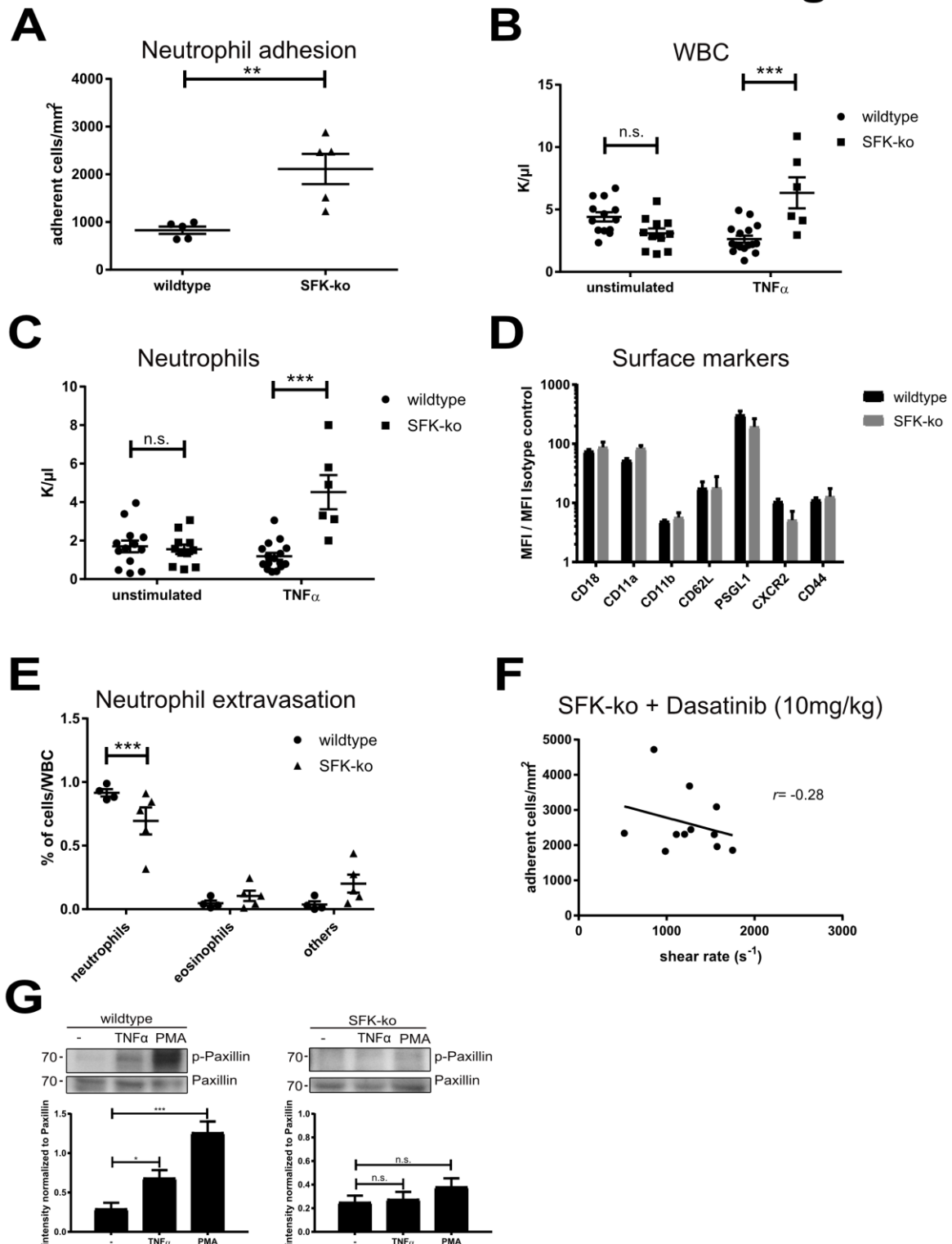


Figure S1. Leukocyte counts and recruitment parameters in wildtype and SFK-ko mice. (A) Number of adherent neutrophils/mm² to postcapillary venules of wildtype and SFK-ko mice. n= 5 wildtype and 5 SFK-ko mice (unpaired Student t test). (B) WBC counts of wildtype or SFK-ko mice with and without TNF_α stimulation. All data is presented as mean ± SEM. * p<0.05, ** p<0.005, *** p<0.001. n.s. = not

significant (2-way ANOVA, Sidak multiple comparison test) (C) Neutrophil counts of wildtype or SFK-ko mice with and without TNF α stimulation. All data is presented as mean \pm SEM. * p<0.05, ** p<0.005, *** p<0.001. n.s. = not significant (2-way ANOVA, Sidak multiple comparison test) (D) Whole blood of wildtype and SFK-ko mice was analyzed for expression levels of neutrophil (Ly6G⁺ cells) surface markers. Mean fluorescent intensities (MFI) are displayed as ratio between MFI of sample and MFI of isotype control. Data is presented as mean \pm SEM (unpaired Student t test). n= 3 wildtype and 3 SFK-ko mice. (E) Relative distribution of extravasated cells in muscle tissue close to a vessel assessed by Giemsa staining. n= 4 wildtype and 5 SFK-ko mice (2-way ANOVA, Sidak multiple comparison test). (F) *In vivo* correlation of shear rates and number of adherent cells in TNF α -stimulated cremaster muscle venules of 2 SFK-ko mice treated for 3h with the tyrosine kinase inhibitor Dasatinib (10mg/kg). (G) Western blot and respective quantitative analysis of Paxillin (Tyr118) (68kDa) phosphorylation after TNF α and PMA stimulation in wildtype and SFK-ko neutrophils plated on fibrinogen. Band intensity was normalized to respective total protein. All data is presented as mean \pm SEM. * p<0.05, ** p<0.005, *** p<0.001. n.s. = not significant. (1-way ANOVA, Dunnett's multiple comparisons test). n= 3 wildtype and 3 SFK-ko mice.

Figure S2

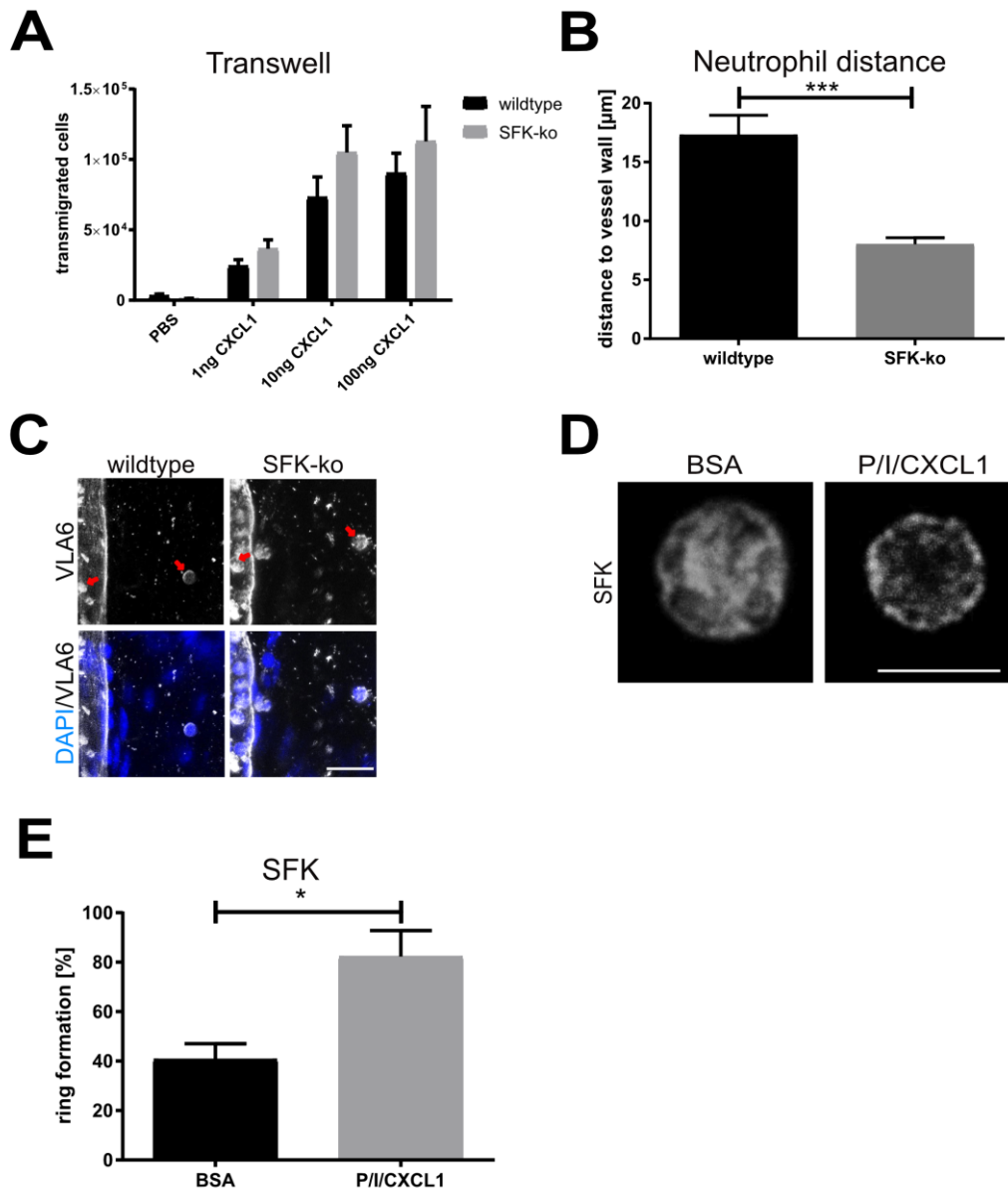


Figure S2. Extravasation mechanisms in wildtype and SFK-ko neutrophils. (A) Number of transmigrated wildtype and SFK-ko neutrophils in a transwell assay using PBS, 1, 10 or 100ng CXCL1 as chemoattractant. Data is presented as mean \pm SEM. $n=3$ wildtype and 3 SFK-ko mice (2-way ANOVA, Sidak multiple comparison test). (B) Quantification of neutrophil distance to the vessel of whole mount stainings as shown in Fig.4B. At least 75 cells were analyzed. All data is presented as mean \pm SEM. * $p < 0.05$.

$p < 0.05$, ** $p < 0.005$, *** $p < 0.001$. n.s. = not significant (unpaired Student t test). (C) Maximum projections of confocal microscopic images of venules in TNF α -stimulated cremaster muscle whole mounts from wildtype and SFK-ko mice. VLA6 antibody staining is displayed in white, cell nuclei were visualized using DAPI (blue). In wildtype mice white arrows point at extravasated neutrophils with ring like VLA6 distribution, while no ring is visible intravascular. In SFK-ko mice, white arrows point at extravasated neutrophils, which show no VLA6 ring formation. Scale bar: 20 μ m. (D) Immunostaining of representative wildtype and SFK-ko neutrophils on BSA- or PECAM-1/ICAM-1/CXCL1-coated coverslips for SFKs analyzed by confocal microscopy. Scale bar: 5 μ m. (E) Quantification of ring formation for SFKs. At least 80 cells from 3 wildtype and 3 SFK-ko mice were analyzed. All data is presented as mean \pm SEM. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$. n.s. = not significant (unpaired Student t test).

Figure S3

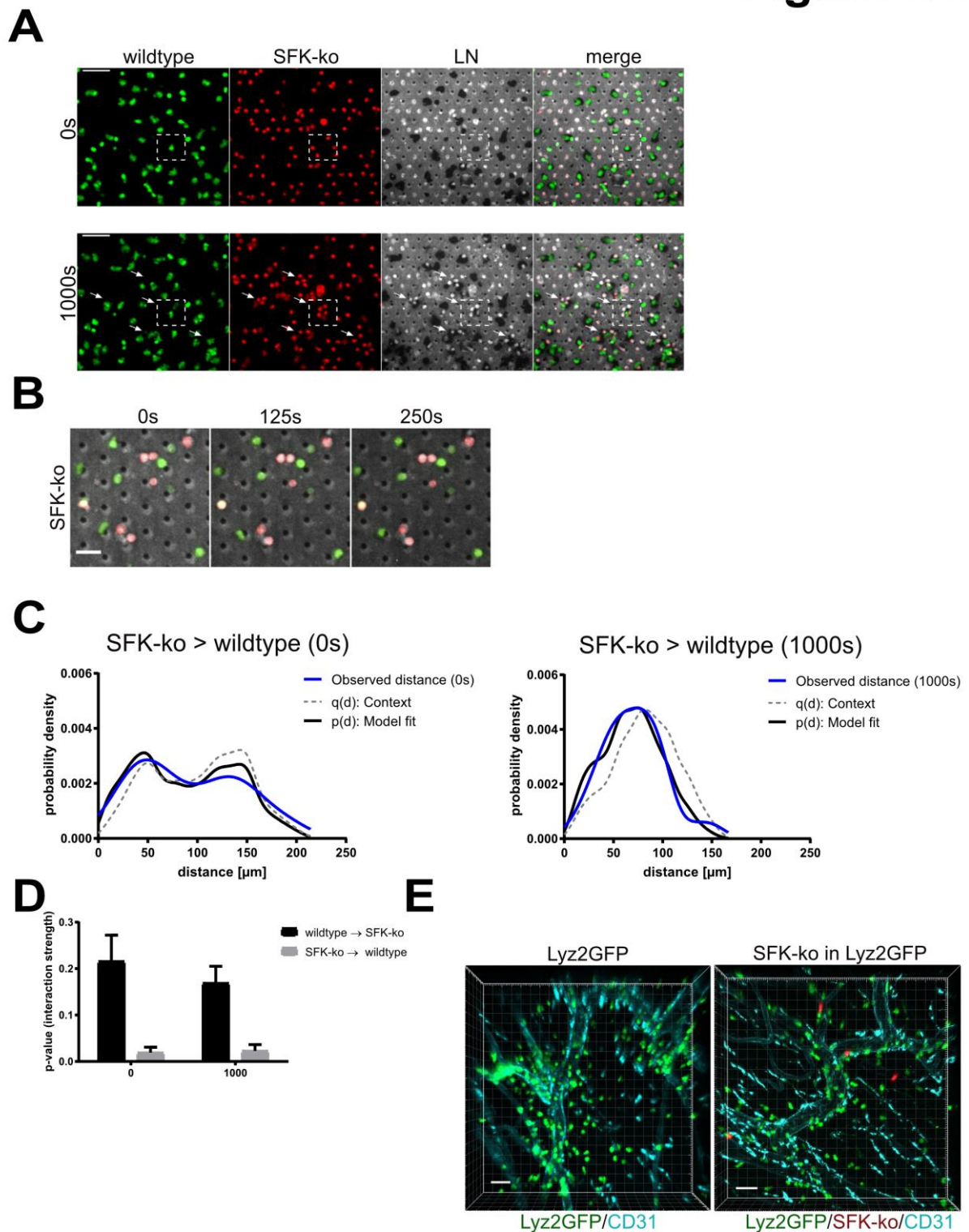


Figure S3. Defective NE mobilization results in diminished LN degradation in SFK-ko neutrophils. (A) Confocal micrographs of wildtype (green) and SFK-ko (red) neutrophils plated on antibody-labeled LN (white) at time point 0 and after 1000s. Dotted square indicates area used for higher magnification in Fig. 5E. Arrows point to areas of SFK-ko neutrophil accumulation around wildtype neutrophils. Scale bar: 50 μm (B) Confocal micrographs of SFK-ko neutrophils labeled with green or red plated together

on antibody-labeled LN (white) at time point 0s, 125s and after 250s. (C) Blue line, observed distance distribution between wildtype and SFK-ko neutrophils. Black line, model fit of the observed distance distribution using a Plummer potential. Grey line, probability density function (context) if SFK-ko cells were distributed randomly and independent of wildtype cells ($n \geq 3$). Data represent mean \pm SEM. * $p < 0.05$, ** $p < 0.005$ (2-way ANOVA, Sidak multiple comparison test). (D) p-values for the interaction strength analysis. (E) Multi photon microscopy of TNF α -stimulated Lyz2^{GFP} or Lyz2^{GFP} mice with 1×10^7 injected deep red labeled SFK-ko neutrophils. Venules were visualized using a CD31 antibody (turquoise). ($n = 4$ mice per group). Scale bar: 30 μ m.

Supplemental References

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Supplemental Movie Legends

Suppl.Mov1. Impaired crawling in SFK-ko neutrophils in microflow chambers. Whole blood from wildtype and SFK-ko mice was perfused through flow chambers coated with E-selectin, ICAM-1 and CXCL1. Using bright field microscopy, crawling cells were recorded under flow conditions during 10 minutes of observation ($\Delta t=3s$). The movies were generated using Fiji software.

Suppl.Mov2. Reduced LFA1 clustering in SFK-ko neutrophils under shear stress. LFA1 clustering in wildtype and SFK-ko neutrophils. Whole blood from wildtype and SFK-ko mice was incubated with a non-blocking LFA1-Alexa 546 antibody and perfused through flow chambers coated with E-selectin, ICAM-1 and CXCL1. Using confocal microscopy, interacting cells were recorded under flow conditions. Images were recorded for 200 seconds ($\Delta t=3s$).

Suppl.Mov3. Digestion of LN by wildtype neutrophils. LN degradation was visualized using time-lapse video microscopy with an average interval of 50s with an upright spinning-disk confocal microscope. Wildtype and SFK-ko neutrophils were stained with CellTracker™ Red CMTPX Dye and CellTracker™ Green CMFDA Dye respectively and in a 1:1 ratio distributed into LN coated ibiPore flow chambers. LN was stained using an Alexa-647-coupled anti-LN antibody. Areas of LN degradation appear black against white LN staining.

Suppl.Mov4. SFK-ko neutrophils accumulate around wildtype neutrophils. Wildtype and SFK-ko neutrophils were stained with CellTracker™ Red CMTPX Dye (SFK-ko) and CellTracker™ Green CMFDA Dye (wildtype) and distributed in a 1:1 ratio into LN (white) coated ibiPore flow chambers. LN was stained using an Alexa-647-coupled anti-LN antibody. Cell migration was visualized using time-lapse video microscopy with an average interval of 50s with an upright spinning-disk confocal microscope over a time period of 1000s.

Suppl.Mov5. SFK-ko neutrophils are unable to extravasate

Multi photon microscopy of TNF α -stimulated SFK-ko x Lyz2^{GFP}. Venules were visualized using a CD31 antibody (turquoise) (n = 4 mice per group). Extravasation was visualized using time-lapse microscopy with an interval of 30s over a time period of 30 minutes.

Suppl.Mov6. SFK-ko neutrophils extravasate in the presence of wildtype neutrophils

Multi photon microscopy of TNF α -stimulated SFK-ko x Lyz2^{GFP} mice with 1x10⁷ injected CellTracker™ Deep Red labeled wildtype neutrophils. Venules were visualized using a CD31 antibody (turquoise) (n = 4 mice per group). Extravasation was visualized using time-lapse microscopy with an interval of 30s over a time period of 30 minutes.

Suppl.Mov7.: Neutrophil transmigration in Lyz2^{GFP} mice

Multi photon microscopy of TNF α -stimulated Lyz2^{GFP}. Venules were visualized using a CD31 antibody (turquoise) (n = 4 mice per group). Extravasation was visualized using time-lapse microscopy with an interval of 30s over a time period of 30 minutes.

Suppl.Mov8.: Injected SFK-ko neutrophils extravasate in wildtype cremaster muscle tissue

Multi photon microscopy of TNF α -stimulated Lyz2^{GFP} mice with 1x10⁷ injected CellTracker™ Deep Red labeled SFK-ko neutrophils. Venules were visualized using a CD31 antibody (turquoise) (n = 4 mice per group). Extravasation was visualized using time-lapse microscopy with an interval of 30s over a time period of 30 minutes.