HETEROTYPIC INTERACTIONS ENABLED BY POLARIZED NEUTROPHIL MICRODOMAINS MEDIATE THROMBO-INFLAMMATORY INJURY

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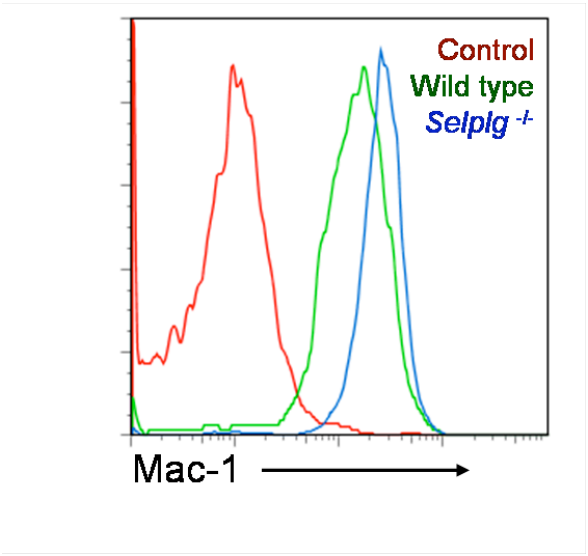
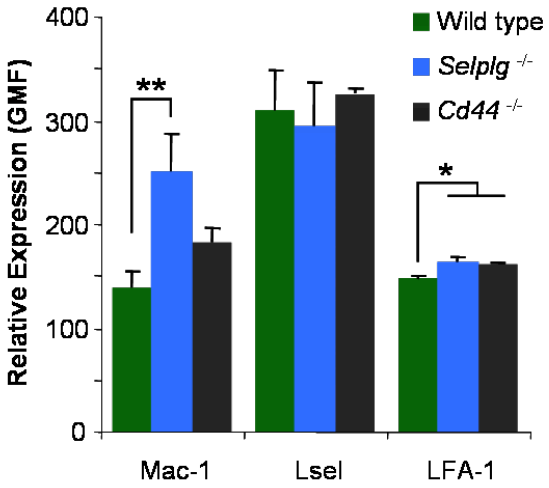
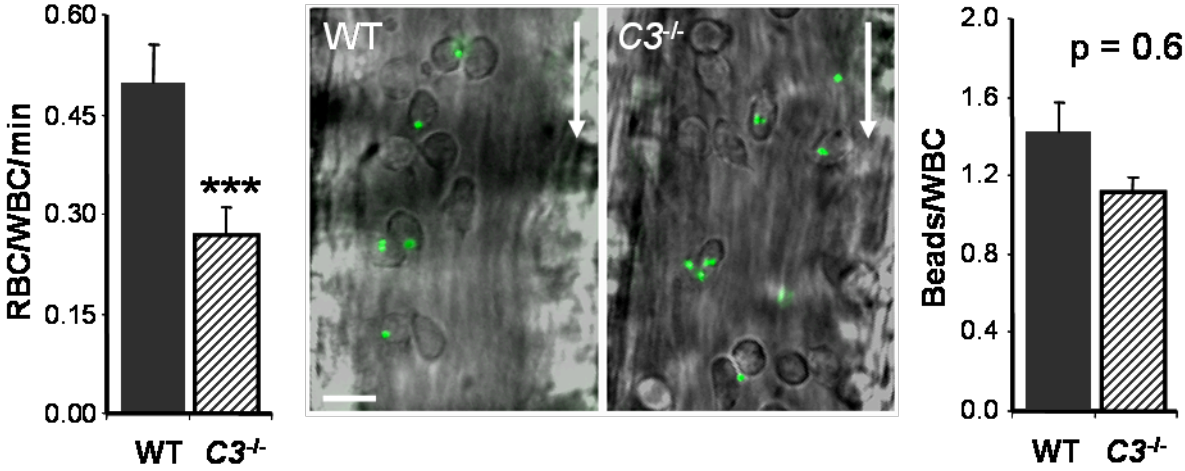
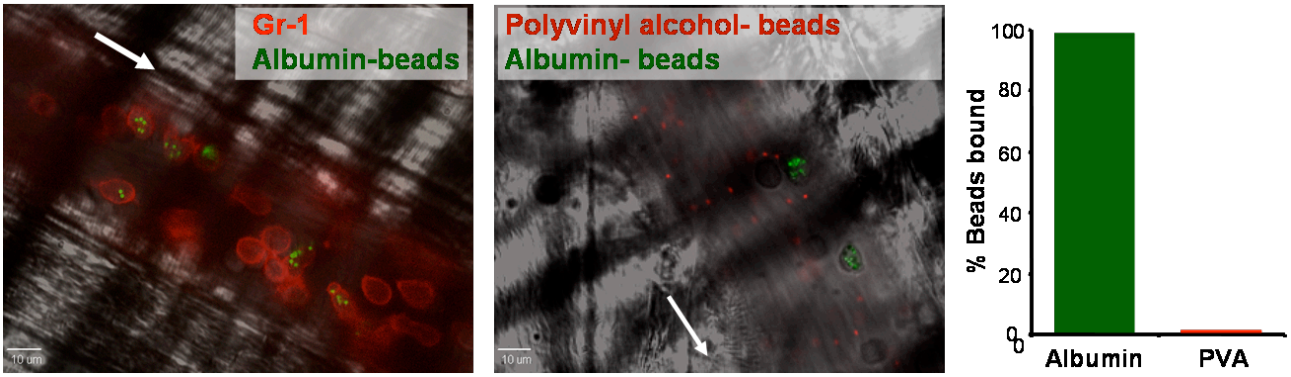




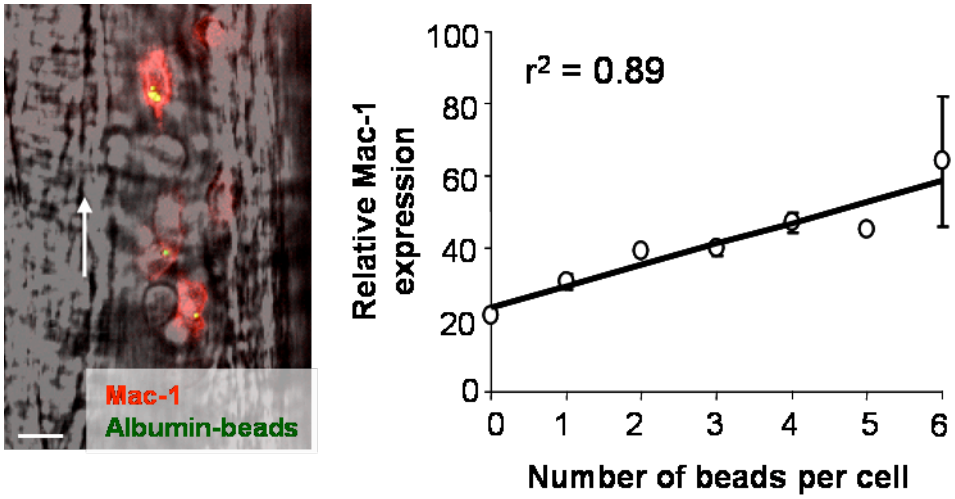
Supplementary Figure 1. Elevated expression of αMβ2 on *Selplg*$^{-/-}$ leukocytes. Circulating leukocytes from wild-type, *Selplg*$^{-/-}$ and *Cd44*$^{-/-}$ mice were stained for αM (Mac-1), L-selectin, and αL (LFA-1) expression after RBC lysis. Staining with APC-conjugated anti-αM (clone M1/70), FITC-conjugated anti-αL (clone M17/4) and PE-conjugated anti-L-selectin (clone MEL-14; all from BD Biosciences) was analyzed on the neutrophil population gated on the basis of side and forward scatter properties by flow cytometry. Representative histogram of αM staining (left panel) and quantification of expression levels (right panel) as the geometric mean of fluorescence (GMF) from 4 (*Cd44*$^{-/-}$) to 8 mice (wild type and *Selplg*$^{-/-}$) per group. *, $p<0.01$; **, $p<0.001$, one-way ANOVA with Tukey's multi-comparison test.



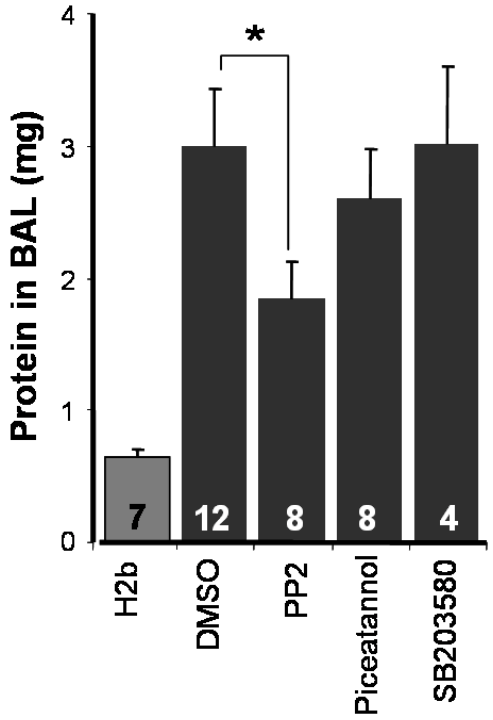
Supplementary Figure 2. Reduced nRBC capture in mice deficient in C3. Interactions of RBC with adherent leukocytes were analyzed in wild-type and *C3*$^{-/-}$ mice (left panel), and mice were subsequently injected with 10^9 albumin-coated fluospheres to evaluate the activation of the integrin $\alpha M\beta 2$ on adherent leukocytes (right panel), as indicated in the Methods section and shown in Figure 4. Middle panels are representative micrographs of fluospheres bound to leukocytes in venules from wild-type and *C3*$^{-/-}$ mice. These experiments indicate that the reduction in RBC-leukocyte interactions in *C3*$^{-/-}$ mice is not due to reduced integrin activity; scale bar = 10 μm; arrows indicate the direction of flow. n = 27-35 venules from 4 mice per group; ***, p = 0.0005, Mann-Whitney test.



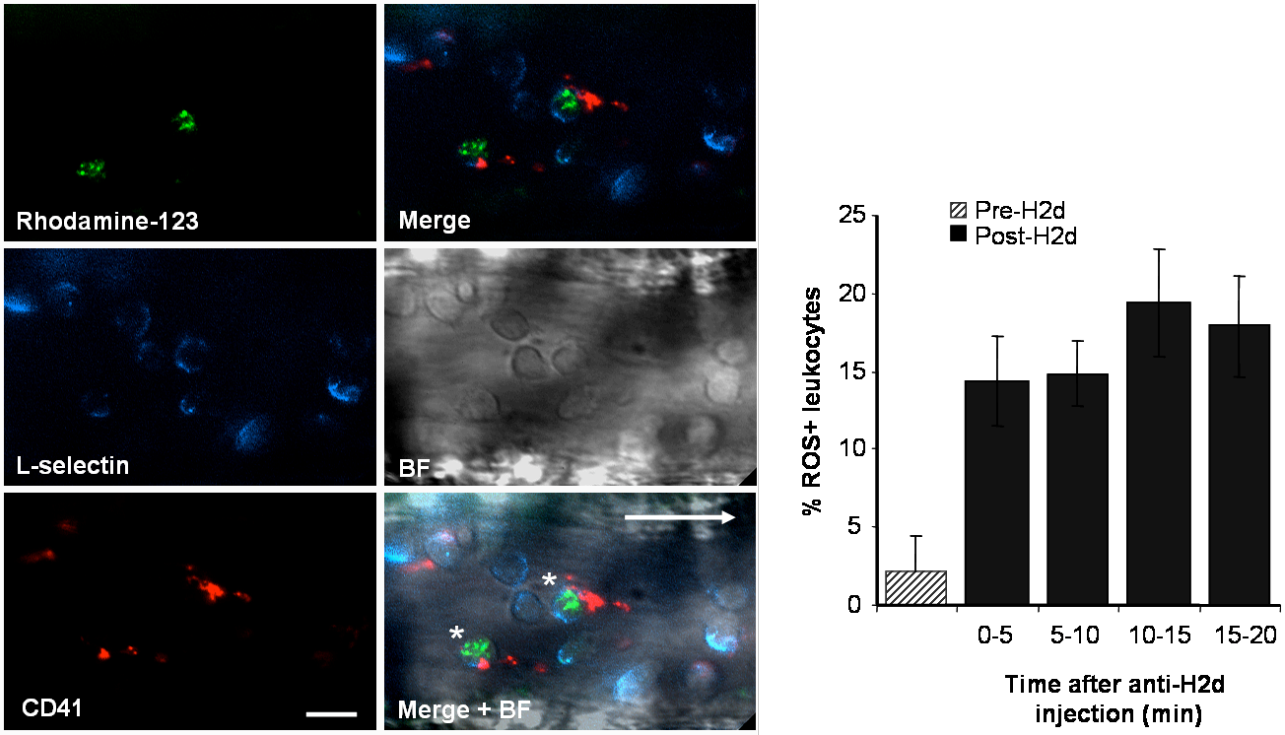
Supplementary Figure 3. Specificity of albumin-coated fluosphere capture *in vivo*. To rule out the possibility that fluospheres might bind to endogenous albumin and be uptaken non-specifically by phagocytes, we coated fluospheres with polyvinyl alcohol (PVA) which prevents binding of plasma albumin. Mice prepared for intravital microscopy were injected with 10^9 albumin-coated fluospheres (green dots) and APC-labeled anti-Gr-1 antibody (red stain; left panel) or 10^9 PVA-coated red fluospheres (red dots; middle panel) 180 min after TNF-α administration. After 10 min, images were acquired in the brightfield, FITC (green) and Cy5 (left panel) or Cy3 (middle panel) channels. Specific binding of fluospheres was restricted to Gr-1+ leukocytes. The number of fluospheres bound to adherent leukocytes (95 beads scored from 2 mice; right bar graph) indicates that the presence of albumin is required for fluosphere capture. PVA-coated fluospheres present in the middle panel were free-flowing in the circulation and did not bind to leukocytes or the endothelium. Scale bars = 10 μm. Arrows indicate the direction of flow.



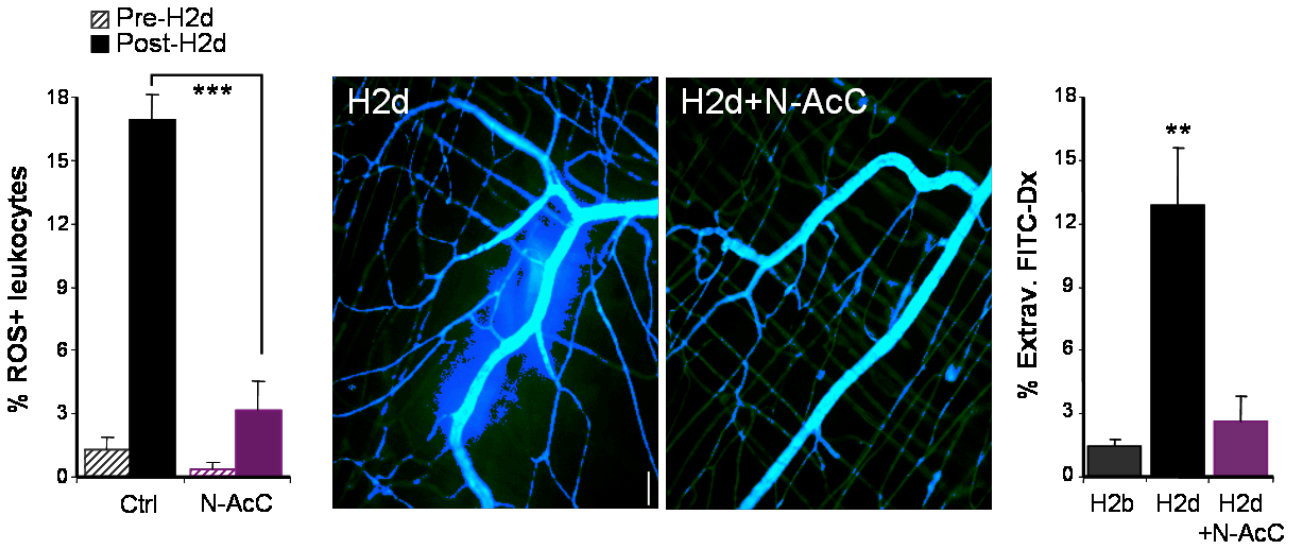
Supplementary Figure 4. Fluosphere capture by adherent leukocytes correlates with the level of αMβ2 expression. Mice prepared for intravital microscopy were injected i.v. with 1 μg APC-conjugated anti-αM, followed by 10^9 albumin-coated green fluospheres 180 min after TNF-α treatment. Images were acquired in the brightfield, FITC (green) and Cy5 (red) channels (left panel). Analysis of relative αMβ2 (Mac-1) expression was performed using the SlideBook software by measuring the mean intensity of Cy5 fluorescence in adherent leukocytes and subtraction of background fluorescence present in an equivalent area in the plasma. The relative intensity associated to each cell was normalized to that of the leukocyte with the highest intensity of fluorescence for each mouse (100% expression). The number of fluospheres bound to the same cells was also counted. Data in the right plot is presented as mean ± SEM, and was derived from the analysis of 93 leukocytes from 18 venules in 2 mice. Arrow indicates the direction of flow. Scale bar = 10 μm.



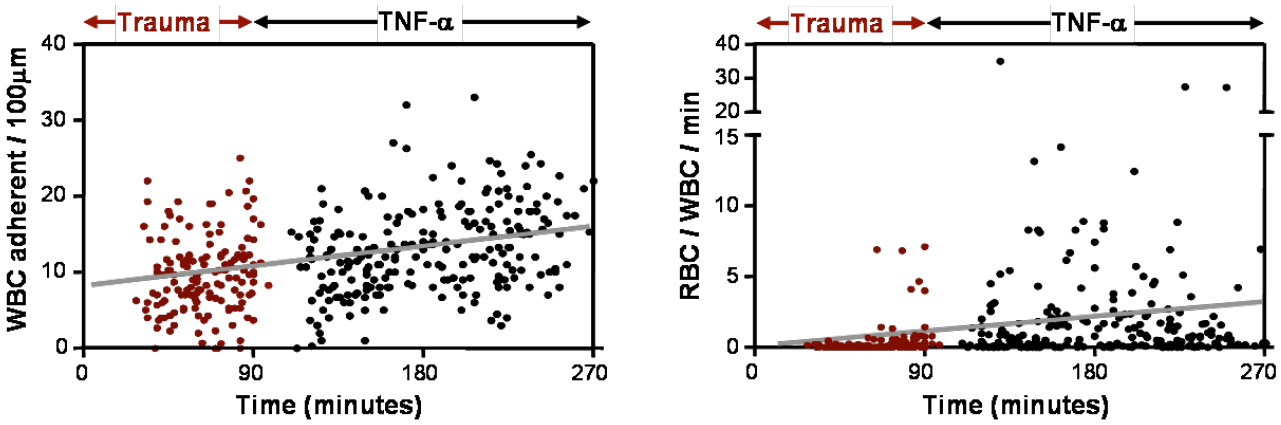
Supplementary Figure 5. Inhibition of Src kinases protects from lung injury. Balb/c mice were treated i.v. with Src inhibitor PP2 (150 μg/kg), piceatannol (1 mg), SB203580 (100 μg), or an equivalent volume of vehicle (dimethyl sulfoxide) 60 min before administration of 3.5 mg/kg anti-H2d antibody. The negative control group (H2b) was injected with the same amount of anti-H2b antibody. After 2h, BAL fluids were harvested and plasma protein content measured. Numbers in bars indicate the number of mice analyzed in each group. Bars represent mean ± SEM. *, $p < 0.05$.



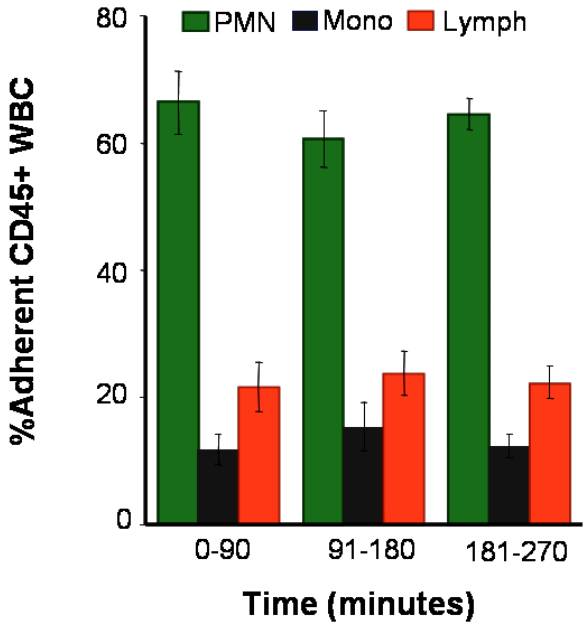
Supplementary Figure 6. ***In vivo*** **detection of reactive oxygen species (ROS) in adherent leukocytes following anti-H2d infusion.** Balb/c mice prepared for intravital microscopy were injected i.v. with 1 μg APC-conjugated anti-L-selectin and 1 μg PE-conjugated anti-CD41 180 min after TNF-α treatment. Mice were also injected with 29 nmoles of dihydroxyrhodamine-123, a probe oxidized to fluorescent rhodamine-123 in the presence of intracellular ROS. Images were acquired in the brightfield (BF), FITC (Rhodamine-123; green), Cy5 (L-selectin; blue) and Cy3 (CD41; red) channels, before and after injection of 3.5 mg/kg anti-H2d antibody. The frequency of ROS-producing leukocytes was calculated by determining the number of rhodamine-123-positive cells (asterisks in the merge + BF micrograph) out of all adherent leukocytes (2 of 11 in this particular image). Scale bar = 10 μm. The arrow indicates the direction of the blood flow. The right bar graph shows the frequency of ROS-producing leukocytes at different time periods after anti-H2d injection. A rapid activation of neutrophils is induced upon treatment and is sustained over time. Data obtained from 5 different experiments.



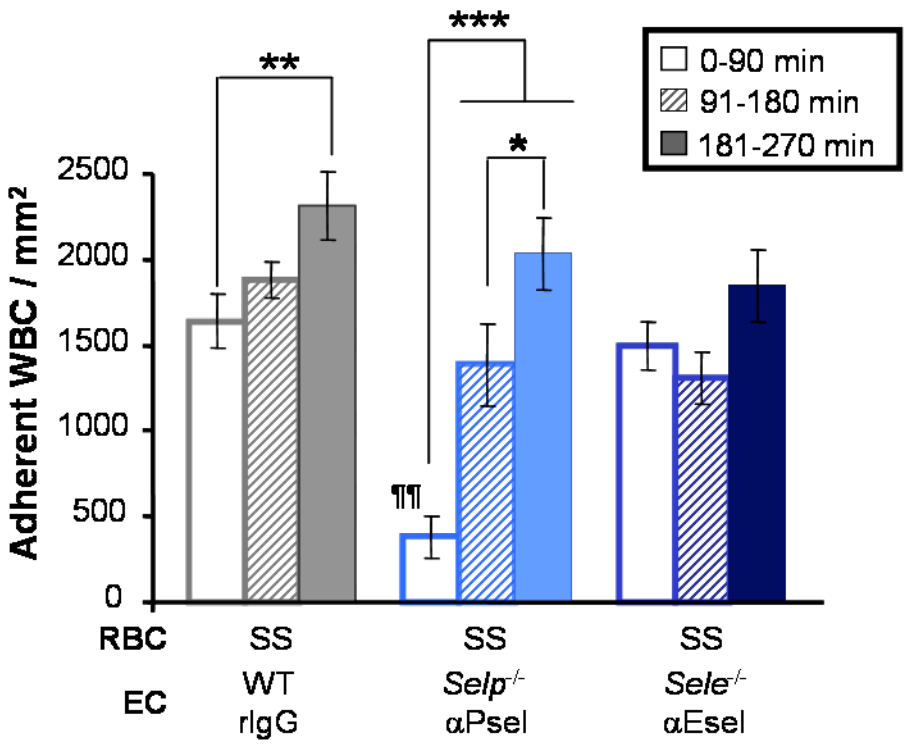
Supplementary Figure 7. N-acetyl-cysteine prevents ROS generation by adherent leukocytes and vascular permeability after anti-H2d infusion. TNF-α-treated mice prepared for intravital microscopy were injected i.v. with 150 mg/kg n-acetyl-cysteine (N-AcC) together with the probe dihydroxyrhodamine-123 to determine ROS production by adherent leukocytes before and after injection of anti-H2d (left panel) as indicated in Supplementary Fig. 5. ***, $p < 0.0001$, Mann-Whitney test; data obtained from the analysis of 20-33 venules from 3-4 mice. FITC-dextran was injected at the end of the experiments to determine vascular permeability (middle micrographs). Images were acquired in the FITC channel (FITC-Dextran; green with blue intensity masking) with a 10x objective. Vascular permeability was determined by quantifying the amount of intravascular and total FITC-dextran signal in the micrographs (right panel). n = 20-28 venular fields; **, $p<0.01$ compared to the other groups, Kruskal-Wallis test with Dunn's multigroup comparison. Vertical scale bar = 50 μm.



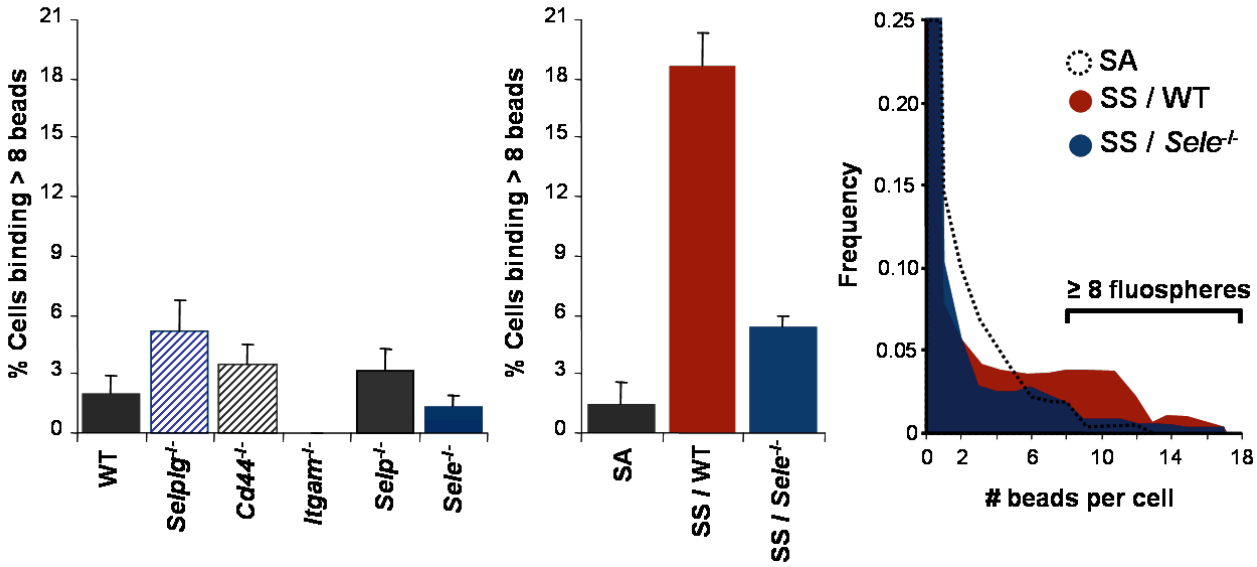
Supplementary Figure 8. Kinetics of leukocyte recruitment and RBC-leukocyte interactions in SCD mice. Analyses were performed before (surgical trauma only) and after TNF-α administration. Left panel shows the number of adherent leukocytes (WBC) in 100 μm-long venular segments. Right panel represents the number of sRBC interactions per adherent leukocyte per minute. Each dot represents one venule recorded at the indicated times.



Supplementary Figure 9. Frequency of adherent leukocyte subsets in venules of sickle transgenic mice. Analyses were performed before (0-90 min) or at different times (91-180 and 181-270 min) after administration of TNF-α. Subsets were identified by high-speed MFIM using fluorescently conjugated CD45, Gr-1 and F4/80 antibodies. No statistical difference was found in the frequencies for the same leukocyte subsets at the different time points. PMN, neutrophils; Mono, monocytes; lymph, lymphocytes.



Supplementary Figure 10. Leukocyte recruitment in venules of wild-type, *Selp*$^{-/-}$ and *Sele*$^{-/-}$ mice reconstituted with hematopoietic stem and progenitor cells from SCD mice. In some experiments, SCD mice were treated with 1 mg/kg of control or blocking antibodies to P- or E-selectin. Cremasteric venules of fully reconstituted mice (100% donor SCD) were analyzed by intravital microscopy at the times indicated. Bars represent mean values ± SEM. n = 7-17 mice per group. *, $p<0.05$; **, $p< 0.01$; ***, $p<0.001$; §, $p<0.01$ compared to values at the same time period in the other groups; one-way ANOVA test with Bonferroni correction. “EC” denotes the endothelial cell phenotype or antibody treatment to inhibit endothelial selectins.



Supplementary Figure 11. Effect of adhesion receptor deficiency and SCD mutation on αMβ2 activity on adherent leukocytes. Wild-type and gene-targeted mice (left panel), or mice transplanted with BM cells from SCD mice (middle and right panels) prepared for intravital microscopy were injected with albumin-conjugated fluospheres as indicated in *Materials and Methods*. The number of fluospheres associated to each adherent intravascular leukocyte was scored and the fraction of cells displaying very high αMβ2 activity (i.e., binding more than 8 beads) averaged and represented as bar graphs. The frequency of leukocytes binding different fluosphere numbers is represented in the histogram (right panel). Data obtained from the analysis of n = 227-507 cells from 4-6 mice per group. The fraction of leukocytes with elevated αMβ2 activity correlates with the frequency of RBC-leukocyte interactions. Bars represent mean ± SEM.

Table 1. Hemodynamic parameters in mice analyzed for RBC-leukocyte interactions

Group	Mice (n)	Venule (n)	Venular diameter (μm)	Centerline Velocity (mm/s)	Shear rate (s^{-1})	RBC/ μL ($x10^6$)
WT (no TNFα)	5	29	36 ± 2	1.24 ± 0.1	368 ± 16	10.4 ± 0.5
WT	8	47	40 ± 1	1.25 ± 0.05	420 ± 18	10.9 ± 0.3
***Selp*$^{-/-}$**	5	31	38 ± 1	1.19 ± 0.1	333 ± 19*	10.0 ± 0.1
***Sele*$^{-/-}$**	7	40	41 ± 1	1.52 ± 0.1*	404 ± 19	10.8 ± 0.3
***Selplg*$^{-/-}$**	7	43	39 ± 2	1.22 ± 0.1	343 ± 43*	10.7 ± 0.1
***Cd44*$^{-/-}$**	5	31	42 ± 2	1.37 ± 0.1	357 ± 19	10.8 ± 0.5
***Itgam*$^{-/-}$**	6	41	37 ± 1	1.31 ± 0.1	384 ± 19	10.7 ± 0.1
***C3*$^{-/-}$**	4	35	37 ± 1	1.2 ± 0.04	348 ± 16	10.7 ± 0.3
WT + DMSO	6	33	41 ± 1	1.14 ± 0.03	304 ± 12	10.2 ± 0.4
WT + PP2	6	34	37 ± 1	1.13 ± 0.04	331 ± 12	10.8 ± 0.3
WT + SB203580	6	43	34 ± 1*	1.07 ± 0.03	341 ± 13	11.4 ± 0.3
WT + Piceatannol	6	44	36 ± 1	1.14 ± 0.03	339 ± 11	11.2 ± 0.2

Group	Mice (n)	Deaths (n)	Venules (n)	Venular diameter (μm)	Centerline velocity (mm/s)	Shear rate (s^{-1})
SS → WT **SS → WT (rIgG control)**	17	5	148	19.2 ± 0.2	0.9 ± 0.1	498 ± 78
SS → *Selp*$^{-/-}$ **SS → WT (αPsel Ab)**	16	3	144	19.8 ± 0.2	1.1 ± 0.1	560 ± 60
SS → *Sele*$^{-/-}$ **SS → WT (αEsel Ab)**	9	0	79	20.1 ± 0.1*	1.4 ± 0.1	757 ± 66

Hemodynamic parameters of venules analyzed for the results presented in Figs. 2 and 6, and Supplementary Fig. 2. Values are mean ± SEM. Shear rates in the sickle transgenic groups (lower table) correspond to the 181-270 min time periods. "Deaths" refers to the number of animals that have died in the course of the experimental period. Note the protection when E-selectin is absent or blocked. *, $p<0.05$ compared to the WT (top table), DMSO (middle table) or SS → WT group (bottom table) in each table; one-way ANOVA with Tukey's multigroup comparison test.

Table 2. Hemodynamic parameters in mice analyzed for fluosphere capture.

Group	Mice (n)	Venules (n)	Venular diameter (μm)	Centerline Velocity (mm/s)	Shear rate (s^{-1})
Wild type	5	37	41 ± 2	1.36 ± 0.2	340 ± 40
***Selp*$^{-/-}$**	4	33	46 ± 2	1.59 ± 0.2	341 ± 46
***Sele*$^{-/-}$**	7	50	42 ± 2	0.93 ± 0.1	243 ± 15
***Selplg*$^{-/-}$**	6	42	40 ± 2	0.81 ± 0.1*	218 ± 17*
***Cd44*$^{-/-}$**	4	30	41 ± 2	0.78 ± 0.1*	207 ± 41*
***Itgam*$^{-/-}$**	4	40	38 ± 2	0.89 ± 0.1	256 ± 15
Scrambl	5	47	47 ± 1	1.76 ± 0.2	416 ± 41
shESL-1	5	48	48 ± 2	1.81 ± 0.2	413 ± 34
SA → WT	4	31	45 ± 2	1.06 ± 0.1	255 ± 26
SS → WT	5	33	45 ± 2	1.71 ± 0.2*	429 ± 53*
SS → *Sele*$^{-/-}$ **SS → WT (αEsel)**	5	38	47 ± 2	1.01 ± 0.1	235 ± 18

Hemodynamic parameters were analyzed from intravital microscopy recordings of venules used for the results shown in Figs. 4 and 6. Presented are means ± SEM of values per venule. *, $p<0.05$ compared to wild type or SA → WT one-way ANOVA with Tukey's multicomparison test.

SUPPLEMENTARY METHODS

Antibodies. Rat anti-mouse monoclonal antibodies anti-CD45 (30-F11), anti-Gr-1 (RB6-8C5), PE-anti-CD45 (30-F11), FITC-anti-LFA-1 (M174), APC- and PE-anti-L-selectin (MEL-14), APC-anti-Gr-1 (RB6-8C5), PE- and APC-anti-αM integrin (M1/70) and rat control isotype (IgG2b,κ) were obtained from BD Biosciences Pharmingen. Rat anti-mouse P-selectin (clone RB40.34; ATCC), F4/80 (ATCC), αM subunit (clone M1/70; ATCC) and anti-E-selectin (clone 9A9) antibodies were purified from supernatants of hybridoma cultures. The anti-MHC-I antibodies directed at the H2b (clone 28-8-6s; mouse IgG2a, κ) or H2d (clone 34-1-2s; mouse IgG2a, κ) haplotypes were purified from hybridoma supernatants (ATCC). Non-specific rat IgG and mouse IgG were obtained from Sigma-Aldrich. AlexaFluor 488, 660, and 555 protein labeling kits were obtained from Invitrogen, and used to label anti-Gr-1, anti-F4/80, and anti-CD45, respectively, as per the manufacturer's instructions.

Generation of mice with knocked-down expression of ESL-1. Generation of lentiviral particles coding for shESL-1 or a control scrambled version was performed as published (reference 4 in main text). shESL-1-transduced cells show a 70% reduction in *Glg1* mRNA levels, a 85% decrease in total ESL-1 protein, and undetectable levels on the cell surface. Donor lineage negative BM cells from wild-type donor mice were transduced with these lentiviral vectors and transplanted into lethally irradiated (1200 cGy, split doses 3h apart) wild-type C57BL/6 recipients. Engraftment of recipient animals was assessed at least three weeks following transplantation by retroorbital bleeding and analysis of GFP^+ leukocytes by flow cytometry.

***In vivo* identification of adherent leukocytes.** Mice were injected via a carotid artery catheter with AlexaFluor555 anti-CD45 (0.12 mg/kg), AlexaFluor488 anti-Gr-1 (0.12 mg/kg), and AlexaFluor660 anti-F4/80 (0.06 mg/kg) Abs, in 300 µL sterile PBS. Images in the FITC, Cy3 and Cy5 channels were recorded 0-90, 91-180 and 181-270 min after muscle exteriorization. Leukocyte identification was performed as indicated previously (reference 25 in main text).

Analysis of platelet interactions. Mice were injected with 1 µg of PE-conjugated anti-CD41 antibody to label intravascular platelets, and 0.02 mg/kg APC-conjugated anti-L-selectin antibody, and images captured as above in the Cy3, Cy5 and brightfield channels. $CD41^+$ particles that remained attached to adherent leukocyte at least the duration of 1 frame (=2 s) were scored as leukocyte-platelet interactions.

Analysis of ESL-1 function. For the analysis of chimeric mice reconstituted with lentiviral-transduced BM cells, four to ten venules per mouse were analyzed 180 to 275 min after TNF-α treatment by acquisition of fluorescence (FITC channel for GFP) and brightfield images using 2 x 2 binning. RBC-leukocyte interactions were analyzed from 1-2 min recordings and scored separately for those mediated by GFP^- and GFP^+ leukocytes. The frequencies of interactions per leukocyte were averaged in each mouse and normalized relative to those found in the GFP^- group.

Analysis of leukocyte microdomains involved in RBC, platelet or fluosphere capture. For the RBC capture experiments, mice were injected with 0.02 mg/kg of a PE-conjugated anti-L-selectin antibody and images captured 180-250 min after TNF-α treatment using the

Cy3 and brightfield channels. For platelet capture experiments, mice were injected with 1 μg of PE-conjugated anti-CD41 antibody to label intravascular platelets, and 0.02 mg/kg APC-conjugated anti-L-selectin antibody, and images captured as above in the Cy3, Cy5 and brightfield channels. The sites of interaction were analyzed visually and scored as mediated by the "leading edge" if RBC or $CD41^+$ platelets were found to interact with the area opposite to the L-selectin-enriched uropod which could be also distinguished by active formation of protruding lamellipodia and by the direction of leukocyte migration. 50% of the cell area on the side of the L-selectin cluster was scored as "trailing edge". For the analysis of the microdomains involved in fluosphere capture, the same procedure was followed except that 10^9 albumin-coated fluospheres were injected when the L-selectin (using an APC-conjugated antibody) clusters were detectable, and images were acquired in the FITC, Cy5 and brightfield channels. The same criteria described above were used for microdomain identification.

Antibody-induced acute lung injury. Balb/c male mice (8-12 week-old) were injected i.v. with 3.5 mg/kg of anti-H2d (clone 34-1-2s) or control H2b (clone 28-8-6s) antibodies. After 2 h, BAL was performed on anesthetized mice by 3 washes with 1 ml PBS each using a 18G needle, and the protein content assessed by the bicinchoninic acid method (BCA; Pierce). In some experiments, mice were pre-treated as follows: rabbit anti-platelet serum (25 μl; i.p. injection; Accurate Chemicals) diluted in 200 μl of PBS, 2 h prior anti-H2d treatment; 1 mg/kg i.v. injection of control or anti-P-selectin, E-selectin or anti-αMβ2 antibodies 5 min before anti-H2d; 150 mg/kg i.v. injection of n-acetyl cysteine 5 min before anti-H2d . Platelet and leukocyte counts from blood samples obtained at the end of the experiment were determined using a hematological Coulter® AC-T diff™ Analyzer

(Beckman Coulter). None of these treatments affected circulating leukocyte counts (not shown). In the TRALI experiments involving intravital imaging of the cremasteric microcirculation, TNF-α was injected intrascrotally to allow the recruitment and imaging of inflammatory leukocytes.

Vascular permeability. To analyze anti-MHC-I-induced vascular damage, Balb/c mice were intravenously infused with 3.5 mg/kg of anti-MHC-I antibodies (to H2d or control H2b) and 1 mg of FITC-Dextran (70 Kd; Sigma-Aldrich) 210 min after TNF-α-treatment. In some experiments, mice were pre-treated with 25 μL rabbit anti-platelet serum to deplete platelets, 1 mg/kg of control, anti-E-selectin or anti-αMβ2 antibodies, or 150 mg/kg of n-acetyl cysteine. Random fields containing small venules were imaged 30 min later using the FITC channel under a LumPlanFI 10x objective, NA 0.30 w. Images were masked over a threshold to quantify all FITC-Dextran-associated fluorescence intensity as well as the signal corresponding only to intravascular spaces. The percentage of extravasated FITC-Dextran was finally obtained using the formula: [(Total intensity – Intravascular intensity) / Total intensity] x 100.

***In vivo* detection of leukocytes producing reactive oxygen species (ROS).** Balb/c mice prepared for MFIM were injected i.v. with 29 nmoles of dihydroxyrhodamine-123 (DHR; Molecular Probes, Eugene, OR). Oxidative activity in ROS-producing leukocytes converts DHR into fluorescent rhodamine-123 (507/529 nm), which can be detected by a bright punctuated pattern in the FITC channel (**Supplementary Fig. 5**). In some experiments, mice were pre-treated with 25 μL rabbit anti-platelet serum to deplete platelets, 1 mg/kg of anti-E-selectin antibody, or 150 mg/kg of n-acetyl cysteine. Images

were captured in the brightfield and FITC channels before and after injection of 3.5 mg/kg anti-H2d antibody, and the frequency of ROS-producing cells calculated out of all adherent leukocytes in each venular segment.

***In vivo* analysis of αMβ2 activity.** Yellow-green or red fluosphere® beads (1 μm in diameter, with excitation/emission of 505/515 nm and 580/605 nm, respectively; Molecular Probes) were incubated with 1 mg/ml bovine serum albumin (Fisher Bioreagents) for at least 2h in phosphate-buffered saline and sonicated for 15 min in a water-bath sonicator (Laboratory Supplies Co.) immediately before use. In some experiments, fluospheres were similarly coated with polyvinyl alcohol (MP Biomedicals). Albumin-coated fluospheres (10^9) were intravenously injected into mice prepared for intravital microscopy as indicated above, 180 min after intra-scrotal TNF-α administration. Images were captured 10 min after injection to allow clearance of fluosphere aggregates which appear in the first minutes. Image analyses were performed using the SlideBook® software.

***In vivo* analysis of αMβ2 activity and expression.** Images were captured for at least 30 s in the brightfield and FITC (for yellow-green fluospheres) channels. Adherent leukocytes were visually identified in the brightfield channel and the number of fluospheres associated to each leukocyte was counted. The average number of albumin-coated fluospheres bound to adherent intravascular leukocytes in a given 100 μm-long venular segment was used as a measure of αMβ2 activity, and was obtained from the formula: Fluospheres / WBC = total number of leukocyte-associated beads per venular segment / number of adherent leukocytes per venular segment. The number of fluospheres bound to individual leukocytes was used to obtain frequency histograms of fluosphere binding per

adherent leukocyte in the different mouse groups (**Fig. 5c-e** and **Supplementary Figures 2 and 10**).

For the analysis of chimeric mice reconstituted with lentiviral-transduced BM cells, 10^9 red fluospheres were intravenously injected 180 min after TNF-α treatment. After 10 min, 5 to 11 venules per mouse were analyzed by acquisition of fluorescence (FITC channel for GFP-expressing cells and Cy3 for red fluospheres) and brightfield images using 2 x 2 binning. Beads bound to leukocytes were analyzed from 1-2 min recordings and scored separately for those mediated by GFP^- and GFP^+ leukocytes. The average number of albumin-coated fluospheres bound to adherent intravascular leukocytes in a 100 μm-long venular segments was obtained from the formula: Beads/WBC = total number of leukocyte-associated beads in all venules analyzed in one mouse / number of adherent leukocytes in all venules analyzed in one mouse. The total number of bound fluospheres and the total leukocytes per leukocyte were averaged in each mouse and normalized relative to those found in the GFP^- group.

For the analysis of $\alpha M\beta 2$ expression and fluosphere binding, mice were injected with 10^9 yellow-green fluospheres and 1 μg APC-conjugated anti-αM antibody 180 min after TNF-α administration. Images were captured in the brightfield, FITC (for fluospheres) and Cy5 (for anti-αM Ab) channels. Areas where adherent leukocytes were present, as determined from brightfield images, were analyzed for the mean intensity level in the Cy5 channel and the number of fluospheres present counted in the FITC channel. The mean intensity level in the Cy5 channel of an area similar to that of an adherent leukocyte was determined in leukocyte-free plasma for each vessel analyzed and subtracted from that of

adherent leukocytes. The relative intensity associated to each cell was normalized to that of the leukocyte with the highest intensity of fluorescence for each mouse (100% expression).

Myeloperoxidase (MPO) activity assay to estimate PMN recruitment in lungs. Mice were sacrificed 2h after anti-H2d antibody injection, the thoracic cavity exposed and a lung lobe excised and weighted. Lung samples were homogenized and sonicated in 1mL of 0.05M potassium phosphate buffer, pH6.0 containing 0.5% hexadecyltrimethylammonium bromide (Sigma) at 4°C and debris removed by centrifugation at 12,000 rpm for 10 min. The supernatant was collected and MPO activity in 10 μL of sample detected by adding 200 μL of tetramethylbenzidine substrate buffer (TMB, Sigma). After 2 min the reaction was stopped by addition of 50 μL of 1M HCl, and absorbance read at 450 nm in a μQuant ELISA plate reader (Biotek Instruments, Inc.). A standard curve using homogenates from known numbers of purified bone marrow PMNs was used to estimate the number of PMN in lungs.

Flow cytometry. Primary blood leukocytes were stained by incubation with 10 μg/mL of fluorescently-labeled antibodies to CD44, αM and L-selectin (all from BD Biosciences), or control antibody. Neutrophils were gated on the basis of low forward-scatter and high side-scatter. Samples were acquired using a FACSCalibur flow cytometer and analyzed using the Flowjo software (Treestar Inc.).

VIDEO CAPTIONS

Supplementary Video 1. nRBC captures are mediated by the leading edge of adherent PMNs. Adherent leukocytes in venules of C57BL/6 mice treated with TNF-α were imaged following the intravenous injection of PE-conjugated anti-L-selectin (red, 0.5 μg) and FITC-conjugated anti-LFA-1 (clone M17/4; green, 1 μg). L-selectin clusters identify the trailing edge of adherent leukocytes. Brightfield images of nRBC interactions with PMNs in inflamed venules were captured 180 min after cytokine administration.

Supplementary Video 2. Platelets interact mostly with leukocyte microdomains at the leading edge. Platelets were labeled by anti-CD41 (red, 1 μg / mouse) and the trailing edge with anti-L-selectin (blue, 0.02 mg/Kg) in a TNF-α-stimulated mouse. Real time is shown in the left upper corner (h:min:s).

Supplementary Video 3. Regional activation of αMβ2 integrin at the leading edge of adherent leukocytes mediates fluosphere capture. TNF-α-treated C57BL/6 mice were injected with PE-conjugated anti-L-selectin (red; 0.5 μg) to label the trailing edge of adherent leukocytes. Fluosphere interactions with adherent leukocytes in inflamed venules were imaged 180 min after cytokine treatment, immediately upon the injection of albumin-coated fluospheres (green) through a catheter placed in the left carotid artery.

Supplementary Videos 4a and 4b. Platelet-WBC interactions are markedly induced by anti-H2d administration in Balb/c mice. Platelets were labeled by anti-CD41 (red, 1 μg / mouse) and the trailing edge with anti-L-selectin (blue, 0.02 mg/Kg). **(a)** Sequence of images just before anti-H2d administration. **(b)** Sequence of images taken after anti-H2d injection. Note the increase in platelet captures by leukocytes and also the interactions of non-labeled RBCs with adherent leukocytes.