SUPPLEMENTARY MATERIALS AND METHODS

Antibodies
The following antibodies were used for immunostaining of cells: mouse anti-vinculin, (Sigma; Deisenhofen; Germany), Cy3-labeled secondary anti-mouse antibody and Cy3-labeled streptavidin (Jackson ImmunoResearch Laboratories, Inc.; Suffolk; UK). Phalloidin Alexa Flour 647 (Invitrogen; Darmstadt; Germany) was used to stain F-actin. The following antibodies were used for flow cytometry: hamster IgG anti-integrin β1, isotype control hamster IgG (all from Biolegend; Uithoorn; Netherlands), rat IgG2a anti-integrin β2, rat IgG2a anti-integrin αL, rat IgG2a anti-CD43, isotype control rat IgG2a, isotype control rat IgG1, rat IgG2b anti-CD44, rat IgG2a anti-CD4, rat IgG2a anti-CD8, rat IgG2b anti-CD3, rat IgG2a anti-CD5, rat IgG2b anti-CD127, hamster IgG1 anti-CD11c (all from BD Biosciences; Heidelberg; Germany); rat IgG2b anti-integrin αM, isotype control rat IgG2b, rat IgG1 anti-CD25, hamster IgG anti-TCRβ (all from eBioscience; Frankfurt; Germany); rat IgG2b anti-integrin αIlb β3 clone JON/A (from Emfret Analytics, Eibelstadt, Germany), rat IgG2b anti-F4/80 (Serotec; Oxford; UK).

The following antibodies were used for Western blotting: mouse anti--glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH; Merck; Darmstadt; Germany); mouse anti-talin-1 (Sigma-Aldrich); rabbit anti-mouse RIAM (Abcam, Cambridge, UK), rabbit anti-mouse Rap1 (SantaCruz Biotechnology, Inc., Heidelberg, Germany), rabbit anti-Lamellipodin (Krause et al., 2004), rabbit anti-CalDAG-GEF (gift from W. Bergmeier, University of North Carolina), rabbit anti-mouse Kindlin-3 (homemade1), mouse anti-human FAK (Tyr397) (BD Biosciences), rabbit anti-mouse FAK (Merck-Millipore), phospho-Tyrosine mouse mAb HRP conjugate (p-Tyr-100), rabbit anti-Pyk2, rabbit anti-phospho-Pyk2 (Tyr402), rabbit anti-phospho-Src (Tyr416) (all Cell Signaling Technology; Leiden, Netherlands), rabbit anti-pan Src (Invitrogen).

Peripheral blood cell counts
Mice were bled from the retroorbital sinus into EDTA-coated tubes. Blood cell counts were determined using a Hemavet 950 analyzer (Drew Scientific Inc., Oxford, CT).

Immunofluorescence
Bone marrow derived macrophages (BMDM) were generated by treating bone marrow cells with R10 medium (RPMI 1640 containing 10% FCS, 25 mM HEPES, 100 U/ml penicillin, and 100 µg/ml streptomycin, 2 mM L-Glu, non-essential amino acids and 50 µM β-mercaptoethanol) supplemented with M-CSF for 5-6 d. Similarly, dendritic cells (DCs) were differentiated from bone marrow cells by culturing them in R10 containing GM-CSF for 10 d. BMDM and DCs were transfected using Mouse Macrophage or Mouse Dendritic Cell Nucleofector Kit from Lonza (Basel, Switzerland) and stained as previously described2. Cells
were imaged at RT with a Leica TCS SP5 X confocal microscope (Leica, Bonn, Germany) using 63 × NA 1.40 oil objective lenses and Leica Confocal Software (LAS AF). Single channels were imaged sequentially. All pictures were processed with Photoshop (Adobe).

Flow cytometry
Cells were incubated with Fc receptor–blocking antibody (Merck-Millipore) in FACS buffer (PBS with 1% FCS and 2 mM Na₂EDTA) and then stained with appropriate fluorophore-conjugated monoclonal antibodies for 30 min on ice. Analysis was performed with a FACSCalibur, a FACSARia or a FACSanto (BD) and data were analysed using FlowJo software.

9EG7 staining
BMDMs and PMNs are treated with with Fc receptor-blocking antibody (Millipore) for 10 min. The cells are kept either unstimulated or activated with 100 ng/ml PMA (Calbiochem) or 2 mM Mn²⁺ and incubated with Alexa647 conjugated anti-CD29 antibody (clone 9EG7, BD Biosciences) for 30 min at 37°C. Cells were analyzed using a FACSARia.

Neutrophil phagocytosis assay
The phagocytosis assay with neutrophils was performed using a pHrodo™ E.coli BioParticles® Phagocytosis Kit for Flow Cytometry (Invitrogen) following the instructions of the manufacturer. To obtain neutrophils mice were bled from the retroorbital sinus and blood was collected into heparin-coated tubes (Sarstedt; Nümbrecht; Germany). Phagocytosis was carried out with 100 µl blood from wt mice either untreated or treated with 2.5 µg β2 integrin-blocking antibody (clone Game-46), RIAM⁻, Talin1⁻ and β2 integrin⁻ mice. Granulocytes were identified by staining with anti-Gr1-PerCP-Cy5.5 antibody (eBiosciences) using a FACSCalibur (BD).

Measurement of oxidative burst
Neutrophils were isolated using the EasySep Mouse Neutrophil Enrichment Kit (STEMCELL technologies; Cologne, Germany). Cells were resuspended in FACS buffer containing 50 µM 2',7'-Dichlorofluorescein Diacetate (H₂DCFDA, Sigma) and incubated for 30 min at 37°C. After loading with H₂DCFDA neutrophils were extensively washed and background fluorescence was measured using a FACSCalibur. Subsequently, neutrophils were treated with tumor necrosis factor-α (TNF-α, 1 µg/ml, Sigma) and fluorescence was measured in 10 min intervals for 1 h.

Adhesion and spreading assays
Adhesion assays were performed as described previously²,³. Non-cell culture treated 96-well plates (Falcon, BD Biosciences) were coated with 0.1 mg/ml fibrinogen (Sigma) in TBS, 4 µg/ml recombinant human intercellular adhesion molecule-1 (ICAM-1) and recombinant
human vascular cell adhesion molecule-1 each (VCAM-1) (both from R&D Systems), 5 µg/ml fibronectin (Sigma) or 5 µg/ml vitronectin (STEMCELL technologies) in coating buffer (20 mM Tris-HCl pH9.0, 150 mM NaCl, 2 mM MgCl$_2$) over night at 4°C. The wells were blocked for 1 h at room temperature (RT) with 1,5% bovine serum albumine (BSA) in phosphate buffered saline (PBS).

PMNs, T cells and macrophages were either left untreated or stimulated with Phorbol-12-Myristate-13-Aacetate (PMA, 100 ng/ml, Calbiochem, Merck-Millipore), 1 µg/ml TNF-α or 1 mM MnCl$_2$. Adhesion assays were also performed with TNF-α-stimulated wt PMNs whose β1 integrins were blocked with 10 µg β1 integrin antibody (clone Ha2/5) per 5 x 10$^5$ cells. Subsequently, 2 x 10$^5$ T cells and 5x10$^4$ PMNs or macrophages in RPMI 1640, 0.1% FCS, 2 5 mM HEPES (GIBCO, Invitrogen) were seeded on indicated ligands for 30 min at 37°C, washed twice with PBS and fixed with 4% PFA in PBS for 15 min. Each experiment was performed in quadruplets. After fixation the number of adherent cells was determined by acquisition of phase contrast pictures from each well and counting adherent and spread cells. Alternatively, adherent cells were stained with 5 mg/ml crystal violet in 2% ethanol for 30 min, washed and the remaining dye was dissolved in 2% SDS and the staining intensity was measured in an ELISA plate reader at 595 nm.

To measure cell spreading cells were seeded for two hours on ligand-coated dishes, fixed with 4% PFA in PBS for 15 min, imaged with an Axiovert 40C microscope (Zeiss; Jena; Germany) equipped with a 40 × NA 0.65 objective (Zeiss) and then spreading area was determined using ImageJ software (US National Institutes of Health).

Coating with immune complexes was done as previously described$^4$. Custom made glass bottom dishes were incubated with 5 mg/ml ovalbumin (Sigma, A5503) in PBS over night at 4°C, followed by the incubation with 25 µg/ml rabbit anti-chicken ovalbumin antiserum (Sigma) for one hour at RT. PMNs were isolated from the BM of wt, RIAM$^{-/-}$ and poly-IC induced talin$^{fl/fl}$ MxCre$^{+}$ mice using the EasySep neutrophil enrichment kit (STEMCELL technologies) and 5 x 10$^4$ cells were plated on the immune complexes for 30 min at 37°C. After washing the slides and fixation of the cells phase contrast pictures were taken and adherent and spread cells were counted.

**Adhesion signaling**

Bone marrow derived macrophages were trypsinized and kept in serum-free RPMI in suspension for 1 h. Then cells were either kept in suspension or plated on a surface coated with 5 µg/ml fibronectin and 4 µg/ml ICAM-1, respectively, for 20 min. Cells were carefully washed with ice cold PBS and lysed in 1% Triton X-100 in TBS, containing protease inhibitors (Roche; Penzberg; Germany) and phosphatase inhibitor cocktails (Sigma). 40 µg of lysates were subjected to 10% SDS-PAGE and subsequent western blot analyses.
**T cell adhesion and lymphocyte homing assays**

10 x 10⁶ splenic cells/ml were cultured in R10 (RPMI 1640 containing 10% FCS, 25 mM HEPES, 100 U/ml penicillin, and 100 µg/ml streptomycin, 2 mM L-Gln, non-essential amino acids and 50 µM β-mercaptoethanol) in non-cell culture treated dishes for 16-20 h in the presence of 5 µg/ml Concanavalin A. The next day, cells were washed and replated in medium containing 5% T-cell culture supplement with ConA (BD Biosciences) and 5% 1 M Methyl-α-D-Mannopyranoside. 24 h later activated, FACS-sorted CD4⁺ high T cells were used for flow cytometry and adhesion assays.

Naive splenocytes from wt, RIAM⁻/⁻, talin⁻⁻/⁻CD4Cre⁺ and β2 integrin⁻/⁻ mice were stained with 0.8 µM CellTrace CFSE dye or 0.3 µM CellTrace Far Red dye (both from Invitrogen) in R10 for 20 min at 37°C. After washing the cells twice, 6 x 10⁶ wt cells stained with one dye were mixed with 6 x 10⁶ wt, RIAM⁻/⁻, talin⁻⁻/⁻CD4Cre⁺ or β2 integrin⁻/⁻ cells stained with the other dye and injected i.v. into recipient mice. 3 mice were injected per genotype and dyes were switched between experiments to control for dye affects. One hour after injection, mice were sacrificed, and axillary, brachial and inguinal lymph nodes and spleen were removed and single cell suspensions were stained for CD4⁺ positive cells. Analyses were performed with a FACSaria (BD Biosciences) and the ratio of CD4⁺ control to knockout cells was determined.

**Intravital microscopy**

500 ng rmTNF-α was injected intrascrotally in a volume of 0.3 ml PBS. Two hours later, mice were anesthetized as previously described⁶ and placed onto a heating pad to maintain body temperature. After intubation and carotid artery cannulation for blood sampling, the cremaster muscle was surgically prepared for intravital microscopy as described⁶. Intravital microscopy was performed on an upright microscope (Olympus BX51, Olympus Corporation, Tokyo, Japan) with a saline immersion objective (40 × NA 0.8). Experiments were recorded via a CCD camera (model CF8/1, Kappa, Gleichen, Germany) on a Panasonic S-VHS recorder. Additional recordings were made with a digital camera (LaVision Biotech, Bielefeld, Germany) and processed with an Imspector software package (LaVision Biotech). The digital recordings were used offline to generate movie clips with ImageJ software. During the experiment, systemic blood samples (10 µl into 90 µl Türck’s solution, Merck) were obtained to assess systemic white blood cell counts.

To investigate chemokine-induced leukocyte arrest *in vivo*, 600 ng CXCL1 were injected per mouse systemically and adherent cells were assessed before and 2 min after chemokine application as described previously⁵. Vessel diameter and vessel segment length of postcapillary venules were measured and centerline red blood cell velocities were obtained in microvessels of the cremaster muscle by a dual photodiode and a digital on-line cross-correlation program (Circusoft Instrumentation, Hockessin, USA) and converted offline to mean blood flow velocities as described⁶. Wall shear
rates were assessed as described\textsuperscript{6}. PMNs not moving for more than 30 s were considered adherent. The number of adherent leukocytes was calculated as number of adherent cells per mm\textsuperscript{2} vessel surface area\textsuperscript{5}.

**Whole-mount histology**
To evaluate extravasated leukocytes in TNF-\(\alpha\)-stimulated cremaster muscles, whole mounts of cremaster muscles were prepared as described\textsuperscript{6}. Briefly, the cremaster muscle was fixed in 4\% PFA for 24 h at 4°C, washed three times in 0.1 M phosphate buffer with 5\% ethanol, and stained with Giemsa (Sigma) at RT for 5 min and developed in 0.01\% acetic acid for contrast. The number of extravasated leukocytes was counted with a Zeiss upright microscope and a 100 × NA 1.3 oil immersion objective (Zeiss).

**Ex vivo flow chamber assay**
An auto-perfused flow chamber assay was performed as previously described\textsuperscript{7}. Briefly, rectangular micro glass capillaries (40 \(\mu\)m x 400 \(\mu\)m cross section; VitroCom; Silsden; UK) were used as flow chambers. They were coated with 20 \(\mu\)g/ml recombinant mouse (rm) E-selectin, 15 \(\mu\)g/ml rmCXCL1 and 15 \(\mu\)g/ml rmICAM-1. Non-specific binding was blocked by incubating the flow chambers with 10\% casein (Sigma) for 2 h at RT. The chambers were then rinsed with PBS and connected to the mouse carotid artery via polyethylene tubing. The flow chamber was fixed under an upright fluorescence microscope (BX51 Wi, Olympus) with a saline immersion objective (20 × NA 0.95, Olympus). Adhesion of leukocytes within the flow chamber was observed for 10 min and recorded via a charge-coupled device camera system (CF8HS; Kappa) on a Panasonic S-VHS recorder. Leukocyte adhesion was assessed after a 10 min perfusion by counting adherent cells in a defined flow chamber segment. Antibody blocking experiments were performed with 30 \(\mu\)g of anti-LFA1 and 30 \(\mu\)g of anti-Mac1 antibodies that were injected into the carotid artery 10 min before assessing adhesion.

**Lung injury model**
LPS-induced acute lung injury was performed as described previously\textsuperscript{8}. In brief, wt and RIAM\textsuperscript{-} littermate mice were exposed to aerosolized LPS (500 \(\mu\)g/ml) from *Salmonella enteritidis* (Sigma) dissolved in 0.9\% saline for 30 min. Neutrophil counts in bronchoalveolar lavage (BAL) and lung tissue (interstitium and pulmonary vasculature) were assessed 4 h after inhalation. 30 min before euthanasia, 5 \(\mu\)l FITC-Ly-6G (Gr1) (eBioscience) were applied by tail vein injection to label intravascular neutrophils. To obtain BAL fluid, lungs were washed via the trachea 5 times with 0.5 ml PBS. Thereafter, the pulmonary vasculature was rinsed with 10 ml ice-cold PBS. The lungs were removed, minced, digested with liberase and passed through a cell strainer to generate a single cell suspension. For FACS analysis BAL fluid and lung homogenate were labelled with antibodies to CD45, CD11b, CD115, F4/80 and Gr1.
Skin inflammation model
Croton oil (Sigma) was diluted to 1% in acetone and topically applied to the ventral and the dorsal sides (20 µl each) of the ears of wt and RIAM−/− littermate mice. After 2 h, mice were sacrificed and spit earflaps were subjected to whole-mount immunostaining. To this end, ear flaps were fixed in 1% paraformaldehyde, blocked with 1% BSA/PBS (PAA Laboratories, GE Healthcare; Munich; Germany), incubated with biotin-labeled anti-Gr-1 (RB6-8C5; BD Biosciences-Pharmingen) to identify PMNs and anti-pan-laminin (L9393; Sigma) to visualize the endothelial basement membrane, and finally anti-rabbit Alexa Fluor 488 (Invitrogen) and streptavidin-Cy3 (Jackson Immunoresearch). Thereafter, tissues were embedded in evanol and representative images were taken with a Zeiss Axio Imager microscope.

REFERENCES
Supplementary Table S1: Microvascular parameters in TNF-α-treated cremaster muscle from wt, RIAM<sup>−/−</sup> and talin-1<sup>−/−</sup> mice (n≥4 mice)

<table>
<thead>
<tr>
<th></th>
<th>Vessel diameter [µm]</th>
<th>Centerline velocity [µm/s]</th>
<th>Wall shear rate [1/s]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt</td>
<td>32.2 ± 1.8</td>
<td>2011 ± 380</td>
<td>1789 ± 338</td>
</tr>
<tr>
<td>RIAM&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>30.0 ± 1.7</td>
<td>2565 ± 300</td>
<td>2126 ± 250</td>
</tr>
<tr>
<td>Talin-1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>39.6 ± 1.1</td>
<td>1400 ± 439</td>
<td>825 ± 289</td>
</tr>
</tbody>
</table>

Supplementary Table S2: Microvascular parameters in non-treated cremaster muscle venules from wt and RIAM<sup>−/−</sup> mice (n≥4 mice)

<table>
<thead>
<tr>
<th></th>
<th>Vessel diameter [µm]</th>
<th>Centerline velocity [µm/s]</th>
<th>Wall shear rate [1/s]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt</td>
<td>30.8 ± 1.8</td>
<td>2108 ± 171</td>
<td>1792 ± 165</td>
</tr>
<tr>
<td>RIAM&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>28.9 ± 1.3</td>
<td>1944 ± 173</td>
<td>1610 ± 164</td>
</tr>
</tbody>
</table>

Supplementary Table S3: Microvascular parameters in TNF-α-treated cremaster muscle from bone marrow chimeras (n≥4 mice)

<table>
<thead>
<tr>
<th></th>
<th>Vessel diameter [µm]</th>
<th>Centerline velocity [µm/s]</th>
<th>Wall shear rate [1/s]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt</td>
<td>30.9 ± 1.2</td>
<td>1511 ± 248</td>
<td>1239 ± 224</td>
</tr>
<tr>
<td>Wt BM in RIAM&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>29.4 ± 0.9</td>
<td>1673 ± 367</td>
<td>1453 ± 351</td>
</tr>
<tr>
<td>RIAM&lt;sup&gt;−/−&lt;/sup&gt; BM in wt</td>
<td>29.3 ± 0.9</td>
<td>1391 ± 123</td>
<td>1184 ± 108</td>
</tr>
</tbody>
</table>
Supplementary Figure S1: Loss of RIAM has no impact on expression of talin-1 and kindlin-3 in different hematopoietic cells. (A-C) Densitometric quantification of RIAM, talin and kindlin-3 protein expression in platelets (A), PMNs (B) and bone marrow derived macrophages (C) from wt, RIAM^{+/+} and RIAM^{-/-} mice after western blot analyses. Intensities were normalized to GAPDH levels and were calculated relative to the expression in wt cells. Values are given as mean ± SD (n≥3).
Supplementary Figure S2: Static adhesion assays with \( \beta_1 \) integrin-blocking antibody treated wt PMNs and \( \beta_2 \) integrin\(^v\) PMNs confirm ligand specificity. (A) Adhesion of wt PMNs to VCAM after treatment with TNF-\( \alpha \) is diminished upon addition of \( \beta_1 \) integrin-blocking antibody. (B,C) \( \beta_2 \) integrin-deficient PMNs poorly adhere to ICAM-1 and fibrinogen after activation with TNF-\( \alpha \) (n=3). (D) Relative amount of fluorescently labeled \textit{E.coli} particles phagocytosed by wt, RIAM\(^v\), talin-1\(^v\), \( \beta_2 \) integrin\(^v\) and \( \beta_2 \) integrin-blocking antibody treated wt PMNs at 4\(^\circ\)C and 37\(^\circ\)C (n=8,8,8,3,3). Values are given as mean \( \pm \) SD. \( p \)-values indicate significant differences and were calculated with Student's \( t \) test.
Supplementary Figure S3: β1 integrin activation in wt, RIAM−/− and talin-1−/− myeloid cells. (A) 9EG7 binding to unstimulated (light gray), PMA treated (red) or Mn^{2+} treated (green) wt, RIAM−/− and talin-1−/− PMNs. (B) 9EG7 binding to RIAM−/− and talin-1−/− macrophages relative to wt macrophages in the presence or absence of 2 mM Mn^{2+}. Values were subtracted by background binding of isotype control and corrected for total β1 integrin surface levels (n=4 independent experiments). Values are given as mean ± SD. p-values indicate significant differences and were calculated with Student’s t-tests.
**Supplementary Figure S4: Adhesion of wt PMNs under flow ex vivo.** Flow chambers were coated with E-selectin, connected to the carotid artery and perfused with whole blood for 10 min. Mice were either left untreated or treated with 30 µg anti-LFA-1 and 30 µg anti Mac-1 blocking antibodies via their carotid catheter 10 min before measurement (n≥3 chambers in 3 independent experiments). Values are given as mean ± SEM.
Supplementary Figure S5: Podosome formation in bone marrow derived macrophages (BMDM) and DCs is not affected by loss or overexpression of RIAM. (A) Vinculin (red) and F-actin staining (phalloidin; blue) of wt and RIAM<sup>−/−</sup> BMDMs expressing EGFP or RIAM-EGFP (green). Scale bar represents 5 μm. (B) FACS blots showing integrin αM and F4/80 expression of wt (green) and RIAM deficient (red) BMDMs at d5 of differentiation. Isotype controls are shown in light and dark gray, respectively. (C) Percentage of wt and RIAM<sup>−/−</sup> BMDMs forming podosomes on a glass surface. Approximately 70 cells were counted in each of 3 different preparations. (D) Average size of podosome clusters formed by wt and RIAM<sup>−/−</sup> BMDMs. Approximately 15 cells were measured in each of 3 different preparations. (E) FACS blot showing CD11c expression of wt (green) and RIAM<sup>−/−</sup> (red) DCs at d10 of differentiation. Isotype controls are shown in light and dark gray, respectively. (F) Percentage of EGFP or RIAM-EGFP expressing wt and RIAM<sup>−/−</sup> DCs forming podosomes. Approximately 70 cells were counted in each of 4 different preparations. (G) Average size of podosome clusters formed by EGFP or RIAM-EGFP expressing wt and RIAM<sup>−/−</sup> DCs. Approximately 15 cells were measured in each of 3 different preparations. Values are given as mean ± SD.