Supplementary Materials and Methods

Immunohistochemistry

The extent of atherosclerosis was assessed in aortic roots by staining for lipid depositions with oil-red-O. Aortic roots were stained with antibodies to Ly6G (1A8, BD Biosciences), Mac2 (Cedarlane), VCAM-1 (429, BD Biosciences), or ICAM-1 (3E2, BD Biosciences). Nuclei were counter-stained by 4',6-Diamidino-2-phenylindol (DAPI). Furthermore, TUNEL staining was performed using In Situ Cell Death Detection Kit, TMR red (Roche) to assess the number of apoptotic/necrotic cells within aortic root sections. Annexin A1 was stained after de-paraffinization of human atherosclerotic plaques or Methanol permeabilisation of aortic root sections from mouse using a rabbit polyclonal anti Annexin A1 antibody (Life Technologies) and a FITC-conjugated anti rabbit antibody (Sigma-Aldrich). Subsequently, tissue-specific antibodies were used to co-stain for Mac2 (Cedarlane), CD68 (eBioscience), endothelial vWF (Abcam), smooth muscle actin (Dako) or Ly6G (BD Biosciences), CD177 (Abnova) and detected by use of Cy3-conjugated anti-rat (Abcam), anti-mouse (Jackson Immuno Research) or anti-sheep (Abcam) antibodies. A Leica DM4000 microscope with a 25/×0.95 water emersion objective (Leica Microsystems) and a Leica DFC 365FX camera were used to capture images. Leica Qwin Imaging software (Leica Ltd.) was employed for image analysis.

The Ethics Committee of the Medical Faculty (RWTH Aachen University) approved the study protocol for the collection of human atherosclerotic plaque samples, and written informed consent was obtained from all participating subjects.

Intravital microscopy

Leukocyte-endothelial interactions along the carotid artery were analyzed in mice having received high fat diet for 4 weeks. Mice were placed in supine position and the right jugular vein was cannulated with a catheter (PE10, Becton Dickinson) for antibody injection. Antibodies to Ly6G (0.5 µg, PE, Biolegend), Ly6C (0.5 µg, AF488, eBioscience), and CD11b (4 µl, 650NC, ebioscience) were administered to label myeloid cell subsets. Antibodies were allowed to circulate for 10 minutes. The left external carotid artery was surgically exposed. Intravital microscopy was performed using an Olympus BX51 microscope equipped with a Hamamatsu 9100-02 EMCCD camera and a 10x saline-immersion objective. For image acquisition and analysis Olympus cell^{r} software was used. Rolling flux was determined as the number of cells passing a reference line perpendicular to blood flow within 30 seconds. Neutrophils were considered adherent when no rolling was observed for at least 30 seconds. For experiments employing Ac2-26 treatment, leukocyte endothelial interactions were recorded prior and 30 minutes after administration of native or boiled Ac2-26 (50 µg via jugular vein catheter). In experiments employing chemokine receptor antagonists, mice received antagonists to CCR2 (RS504393, 5 mg/kg), CCR5 (DAPTA, 1 mg/kg), CXCR2 (SB225002, 5 mg/kg, all Tocris Bioscience) alone or in combination (i.p., in 200 µl vehicle) 30 minutes prior to exposure of the carotid artery. Then, adhesion of myeloid cell subsets was recorded and mice received a bolus of Ac2-26 (50 µg via jugular vein catheter). A second recording was made 30 minutes after Ac2-26 treatment.

Integrin activation assay

Murine peripheral blood cells were drawn retro-orbitally from wild type or *Fpr2*$^{-/-}$ mice, erythrocytes were lysed (lysis buffer: 150 mM NH_4Cl; 10 mM $KHCO_3$; 0.1 mM diNaEDTA, pH 7.4) and leukocytes were suspended in Hanks Balanced Salt Solution containing 1 mM CaCl and MgCl (Invitrogen) and 0.5 % BSA (Sigma). Cells were exposed to CCL2 (5 µg/ml), CCL5 (5 µg/ml), CXCL1 (5 µg/ml; all Peprotech), and LTB4 (1 µg/ml, Santa Cruz) or an equal volume of buffer, in the presence of ICAM-1/Fc (5 µg/ml, R&D Systems) or of VCAM-1/Fc (5 µg/ml, R&D Systems) and PE-conjugated anti-human IgG1 (1 µg/ml; Fc-specific, Southern Biotechnology) for 5 minutes at 37 °C. After washing, cells were labeled with antibodies to CD45, CD115, Ly6G and Gr1 to identify classical monocytes and neutrophils. Binding of ICAM-1 or VCAM-1 was measured by flow cytometry.

Measurements of chemokine receptor and integrin expression

Murine peripheral blood cells were drawn retro-orbitally from wild type mice, erythrocytes were lysed and leukocytes were suspended in HBSS. Cells were stained with antibodies to CCR1 (FITC, R&D sytems), CCR3 (APC, eBioscience), and CCR5 (PE, eBioscience). After washing, cells were labeled with antibodies to CD45, CD115, Ly6G and Gr1 to identify classical monocytes and neutrophils.

Chemokine receptor expression was measured by flow cytometry. For measurement of total surface β_1 and β_2 integrins leukocytes were exposed to CCL5 (5 µg/ml, Peprotech) or an equal volume of buffer. After washing, cells were labeled with antibodies to CD18 (eBioscience) and CD29 (eBioscience). Integrin expression was assessed by flow cytometry.

Cholesterol measurements

Cholesterol and triglyceride levels in mouse serum were quantified using enzymatic assays (Roche and BioTrend) according to the manufacturer's protocol.

Annexin A1 ELISA

Commercially available ELISAs for mouse Annexin A1 were performed in accordance with the manufacturer's protocol (Cloud Clone Corp.).

LFA-1 clustering

Clustering of the integrin $\alpha L\beta_2$ was investigated as described before (Kempf et al., 2011). Briefly, isolated murine or human neutrophils and monocytes were incubated with Ac2-26 at 37°C for 30 minutes and consecutively stimulated with CCL5 for 5 minutes. After fixation with 4% PFA/PBS, cells were immobilized on Poly-L-Lysine-coated glass slides (Lab-Tek 2 chamber slides, Thermo Scientific) and incubated with an Alexa488-labeled anti-LFA1 antibody (clone M17/4, Biolegend). Human neutrophils or monocytes were stained with and uncoupled anti-LFA1 antibody (clone TS2/4, Biolegend) and secondary staining was performed with an Alexa488-conjugated anti.ouse antibody (Life Technologies). Images were acquired using a confocal spinning disc microscope (CellObserver SD, Zeiss).

Assessment of β_2 integrin conformation

We incubated human neutrophils and monocytes ($5x10^5$) with monoclonal antibody clone 24 (mAb24) or mouse IgG1 (clone NCG01, Dianova) for 15 min at 37 °C in 50 µL HBSS (pH 7.4) containing 1 mM Ca^{2+}, 1 mM Mg^{2+}, 0.1% glucose, and 10 mM HEPES in the presence or absence of CCL5 and Ac2-26. We detected antibody binding by flow cytometry using FITC-conjugated goat antibody to mouse IgG (Dianova). In some experiments, isolated human neutrophils or monocytes were preincubated with 1 µM Rap1-WT or Rap1-CA Tat peptides at 37°C for 30 minutes prior to stimulation.

Rap1 activation assay

Rap1 activation in neutrophils following chemokine stimulation was investigated as described previously, with some modifications (Kempf et al. 2011). Briefly, isolated murine or human neutrophils and monocytes were incubated with Ac2-26 at 37 °C for 30 minutes and consecutively stimulated with CCL5 for 5 minutes. After lysis with ice-cold lysis buffer (50 mm TrisHCl pH 7.4: 500 mM NaCl; 1 % NP40; 10 % glycerol; 2.5 mM $MgCl_2$; 10µg/ml PEFA-Block; 25 mM NaF; 1 mM Vanadate and 1 mM PMSF, 1 % Roche HALT protease inhibitor cocktail), GTP-bound Rap1 (Rap1-GTP) was precipitated from whole cell lysates using GST-Ral beads. Precipitated proteins were separated using SDS-PAGE, transferred to a PVDF membrane and immunoblotted against Rap1. To determine the level of total Rap1, a small portion of the whole cell lysate was mixed with SDS sample buffer and separated by SDS-PAGE. Rap1 was detected using a rabbit polyclonal antibody (Santa Cruz).

Ca^{2+} mobilization

Mouse neutrophils were loaded with FLIPR Calcium 5 dye (Molecular Devices) for 1 h at 37 °C and suspendend at a final concentration of 10^6 cells/ml. Neutrophils were allowed to sediment on a Nunc™ Lab-Tek™ chambered coverglass and then treated with CCL5 (5 µg/ml). In same conditions cells were pre-incubated for 30 minutes with Ac2-26. Using a live cell imaging microscope (Olympus MT10 and Cell^M&Cell^R Imaging software), fluorescence images (at 488 nm) were obtained at 1-s intervals for up to 40 s after stimulation. Regions-of-interest, ROIs, were drawn around individual cells for measurement of fluorescence intensities (one ROI/cell). Fluorescence intensities of ROIs were determined using Image J software.

Flow adhesion assay

Human umbilical vein endothelial cells (HUVEC) (PromoCell) seeded on collagen-coated dishes and grown in endothelial growth medium (PromoCell) were activated with TNF (10 ng/ml, 12 h, Peprotech) prior to experimentation. Neutrophils were isolated by density gradient centrifugation using polymorphprep (Axis Shield) and monocytes were isolated by magnetic bead isolation (Monocytes Isolation Kit II, MACS Miltenyi Biotec Inc.). Isolated cells were labeled with Calcein AM (Invitrogen) and incubated with vehicle or Ac2-26 at indicated concentrations prior to performance of the flow chamber assay. Cells were perfused at 2.5 dyne/cm2 and adherent cells were quantified in 15 randomly chosen fields.

Surface plasmon resonance

Interaction between Ac2-26 and FPR2 were studied by Surface Plasmon Resonance on a Biacore X100 system (GE Healthcare). Ac2-26 was immobilized on a CM4 sensor chip at a level of 400 response unit (RU) by amine coupling. Human FPR2 incorporated into liposomes (Abnova) was used as analyte and diluted in a HBS-N running buffer (0.1M HEPES, 1.5M NaCl pH 7.4). Each experiment was performed with a flow of 10µl/min with running buffer.

Competitive Ac2-26 binding assay

Human embryonic endothelial (HEK) 293 cells overexpressing either mouse FPR2 or human FPR2 were incubated with increasing concentrations of unlabeled Ac2-26 (0.1 nM to 10 µM) and [125I]-Tyr–Ac2-26 (50 nM, 1 h, 4 °C) (Phoenix Pharmaceuticals, Karlsruhe Germany). Cells were then transferred on to Whatman GF/C glass microfiber filters (Kent, U.K.) and unbound tracer was washed off using ice-cold PBS (pH 7.45). Filters were then transferred into tubes and radioactivity measured using a gamma counter.

Statistics

All data are expressed as mean±SD. Statistical calculations were performed using GraphPad Prism 5 (GraphPad Software Inc.). After calculating for normality by D`Agostino Pearson omnibus test, unpaired Student's t-test, paired Student's t-test or nonparametric Mann-Whitney U-test, One-way ANOVA, Friedman-test, Wilcoxon matched-pairs signed rank test or Kruskal-Wallis test with post-hoc Dunn test were used as appropriate. p-values < 0.05 were considered significant.

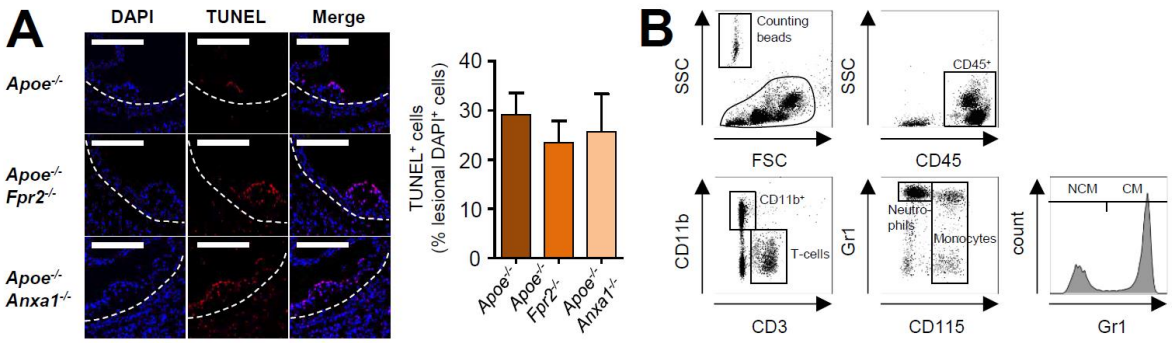
	Apoe$^{-/-}$	*Apoe*$^{-/-}$*Fpr2*$^{-/-}$	*Apoe*$^{-/-}$*Anxa1*$^{-/-}$
Blood cell counts		**(x10^5/ml)**	
Neutrophils	12.83 +/- 6.08	14.10 +/- 6.09	15.47 +/- 4.55
Total monocytes	4.55 +/- 2.11	3.98 +/- 1.88	5.29 +/-1.43
Classical monocytes	3.51 +/- 1.71	3.72 +/- 2.04	4.13 +/- 1.23
Non-classical monocytes	0.80 +/- 0.46	0.51 +/- 0.25	0.70 +/- 0.47
T-cells	7.75 +/- 2.39	5.86 +/- 1.31	9.19 +/- 1.54
Lipid levels		**(mg/dl)**	
Cholesterol	839.50 +/- 98.40	810.60 +/- 138.90	1297.00 +/- 370.3*
Triglyceride	143.20 +/- 54.61	162.10 +/- 63.62	205.90 +/- 69.15*

Online Table I: White blood cell counts and plasma lipid levels in *Apoe*$^{-/-}$, *Apoe*$^{-/-}$*Fpr2*$^{-/-}$, and *Apoe*$^{-/-}$*Anxa1*$^{-/-}$ mice. Absolute counts of indicated circulating leukocyte subsets, serum cholesterol, and triglyceride levels were measured after four weeks of high fat diet. All data are presented as mean ± SD. Experiments were performed three times independently with a total of 15 mice. Data were analyzed with one way ANOVA with Dunnett post test.

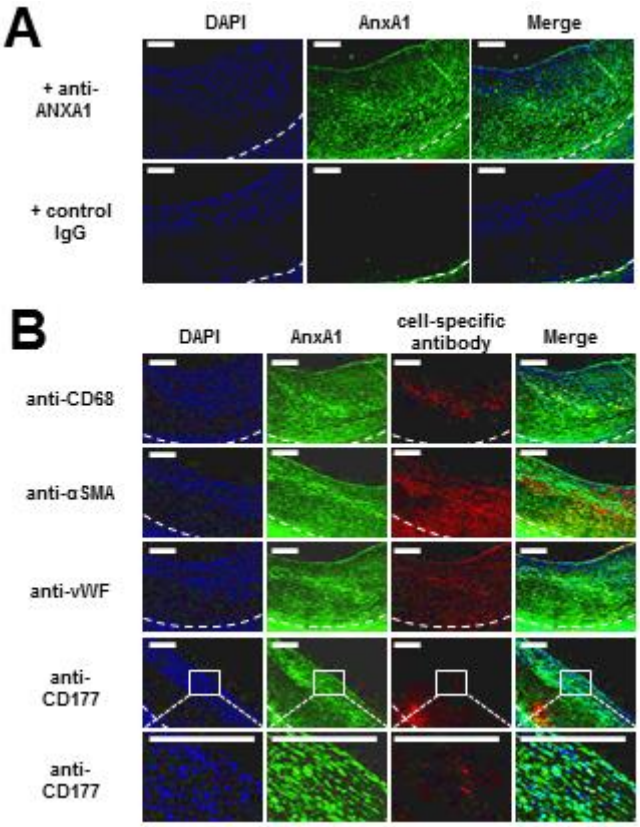
	Apoe$^{-/-}$ vehicle	*Apoe*$^{-/-}$ Ac2-26
Blood cell counts	**(x10^5/ml)**	
Neutrophils	10.44 +/- 4.55	8.02 +/- 4.30
Total monocytes	2.67 +/- 0.75	2.65 +/- 1.43
Classical monocytes	2.26 +/- 0.59	2.07 +/- 1.08
Non-classical monocytes	0.44 +/- 0.28	0.58 +/- 0.46
T-cells	5.49 +/- 1.58	4.87 +/- 2.45
Lipid levels	**(mg/dl)**	
Cholesterol	936.00 +/- 219.99	748.66 +/- 92.91
Triglyceride	123.19 +/- 46.28	129.49 +/- 48.05

Online Table II: White blood cell counts and plasma lipid levels in *Apoe*$^{-/-}$ mice treated with Ac2-26 or vehicle. Absolute counts of indicated circulating leukocyte subsets, serum cholesterol, and triglyceride levels in *Apoe*$^{-/-}$ mice receiving Ac2-26 (3x/week, 50 µg i.p./injection) or vehicle control during 4 weeks of HFD. Data were analyzed with Mann-Whitney test.

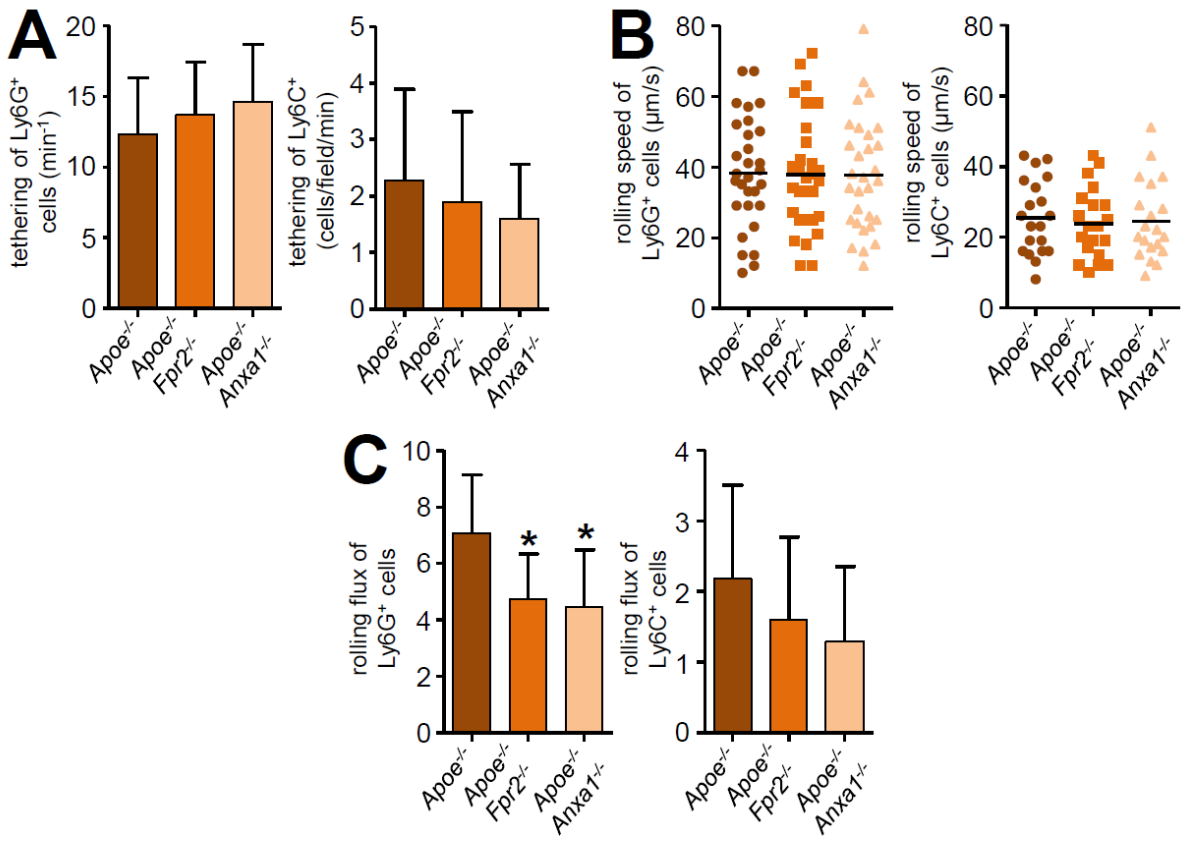
Figure Legends



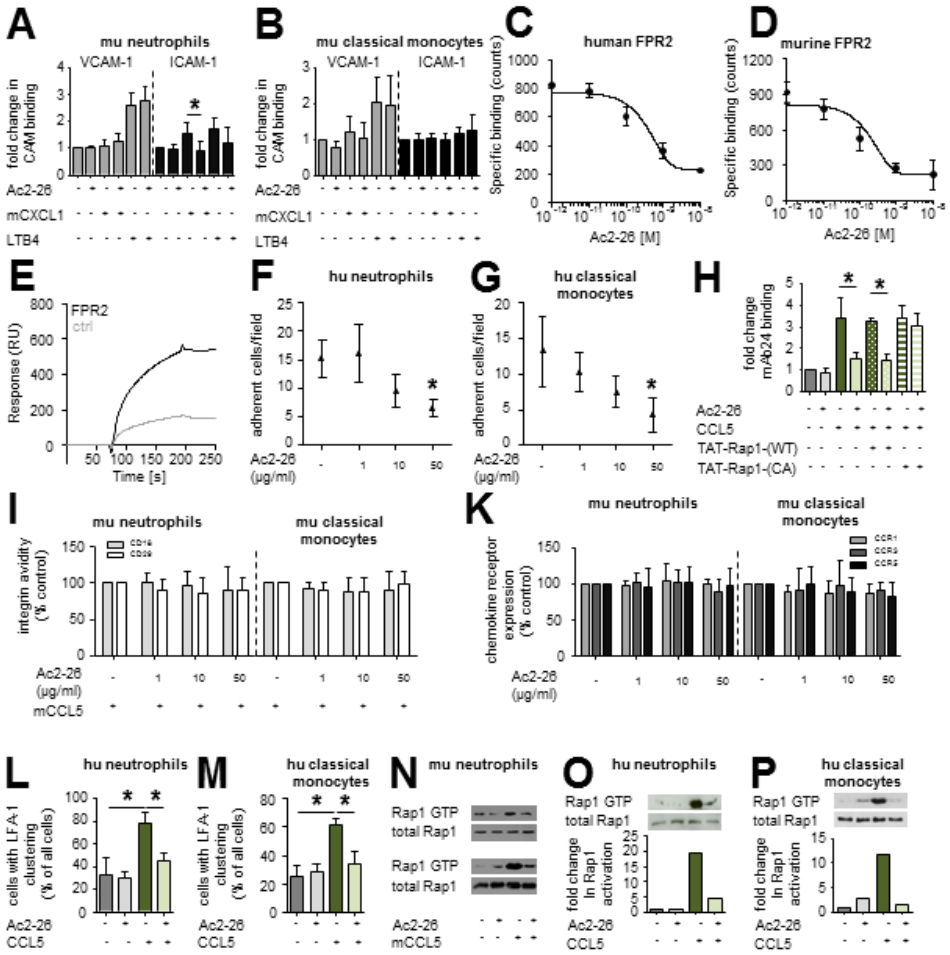
Online Figure I: Lack of FPR2 or its ligand Annexin A1 does not affect accumulation of apoptotic cells in early atherosclerosis. A: *Apoe*$^{-/-}$, *Apoe*$^{-/-}$*Fpr2*$^{-/-}$, and *Apoe*$^{-/-}$*Anxa1*$^{-/-}$ mice were fed a high fat diet for 4 weeks. Apoptotic cells were quantified by TUNEL staining. Displayed are representative images and quantification. All data are presented as mean ± SD. Experiments were performed three times independently with a total of 15 mice. Data were analyzed with one way ANOVA with Dunnett post test. **B:** Display of gating strategy for identification of circulating myeloid cell subsets. NCM, non-classical monocytes; CM, classical monocytes.



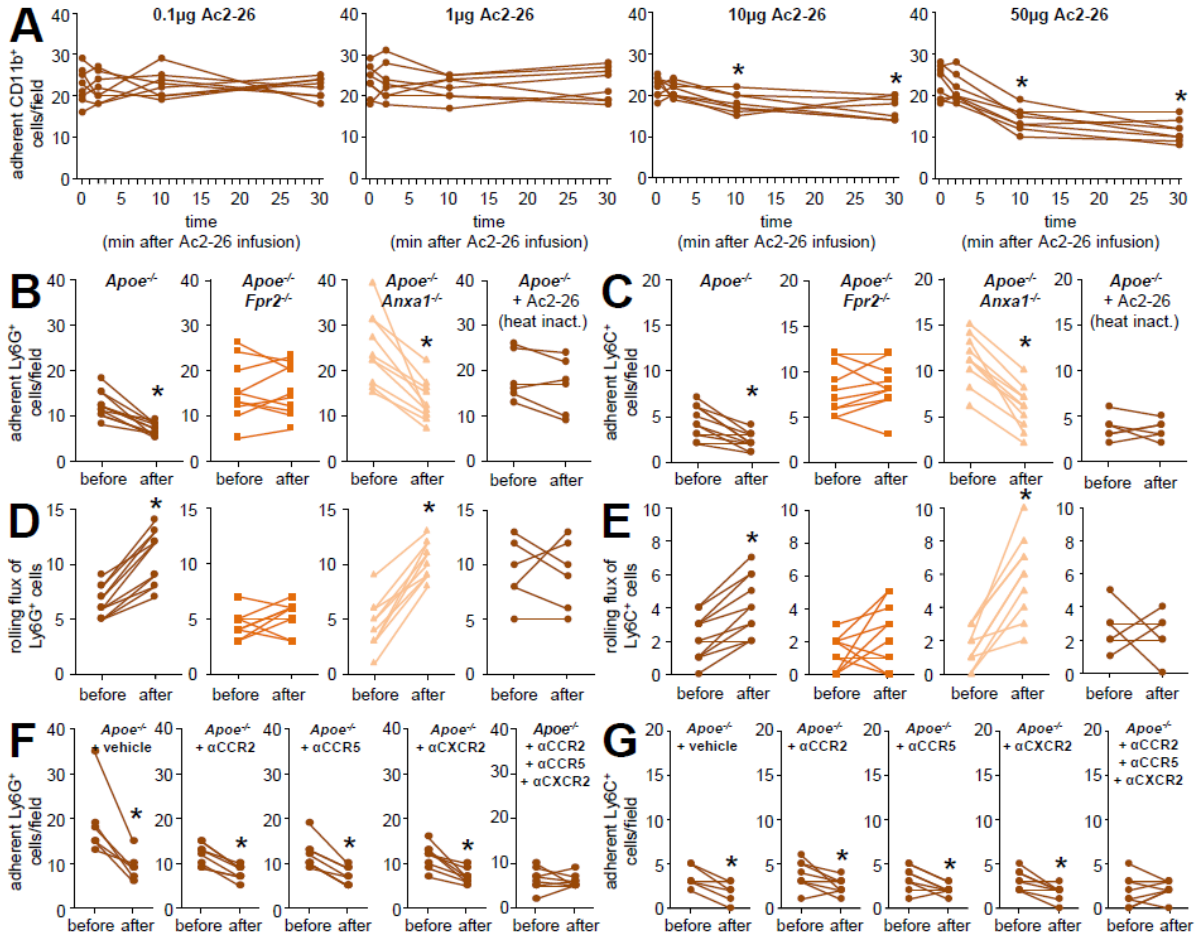
Online Figure II: Annexin A1 is present in lesional myeloid cells and the endothelium. Human atherosclerotic specimens were stained with an isotype control antibody (A), or an Annexin A1 antibody together with antibodies identifying macrophages (anti-CD68), smooth muscle cells (anti-αSMA), endothelial cells (anti-vWF), or neutrophils (anti-CD177) (B). Scale bar represents 100 µm.



Online Figure III: Annexin A1-FPR2 axis does not impact on early interactions between myeloid and endothelial cells. A: *Apoe*$^{-/-}$, *Apoe*$^{-/-}$*Fpr2*$^{-/-}$, and *Apoe*$^{-/-}$*Anxa1*$^{-/-}$ mice were fed a high fat diet for 4 weeks. Intravital fluorescence microscopy of the carotid artery for assessment of luminal leukocyte endothelial interactions. Myeloid cell subsets were identified by i.v. injection of antibodies to Ly6G (PE, 0.5 µg) or Ly6C (AF488, 0.5 µg). Displayed are quantification of tethering (A), rolling speed (B), and rolling flux (C) of Ly6G^{+} (left in each panel) and Ly6C^{+} (right in each panel) cells. All data are presented as mean ± SD. Experiments were performed three times independently with a total of at least 15 mice. Data were analyzed with one way ANOVA with Dunnett post test.



Online Figure IV: Ac2-26-inflicted reduction of integrin activation is independent of integrin expression and chemokine receptor expression but depends on inhibition of Rap1 activation. A/B: Neutrophils (A) and classical monocytes (B) from C57Bl/6 mice were treated with murine CXCL1 (5 µg/ml) or LTB4 (1 µg/ml), and binding of soluble VCAM-1-Fc or ICAM-1-Fc was assessed by flow cytometry. Effect of Ac2-26 was determined by pretreatment for 30 min prior to chemokine stimulation. **C/D:** Binding affinity of Ac2-26 was investigated by incubation of Ac2-26 (0.1 nM to 10 µM) with human FPR2 (C) or mouse FPR2 (D) overexpressing HEK293 cells. **E:** Representative surface plasmon resonance sensorgrams of binding of FPR2 expressed in proteoliposomes to immobilized Ac2-26 on a CM4-sensorchip. Empty proteoliposomes were used as control. **F/G:** Flow adhesion of human neutrophils (F) or monocytes (G) to TNF-activated endothelial cells in presence of indicated concentrations of Ac2-26. **H:** β2 integrin activation assessed by mAb24 binding and flow cytometry in human monocytes incubated with CCL5 and preincubated with Ac2-26. Where indicated, neutrophils were pretreated with wild type (WT) or constitutively active (CA) Rap1-Tat peptides. **I:** Dose-dependent effect of Ac2-26 on surface expression of CD29 and CD18 on neutrophils and classical monocytes in presence of CCL5. K**:** Dose-dependent effect of Ac2-26 on surface expression of CCL5 receptors CCR1, CCR3, and CCR5 on neutrophils and classical monocytes. **L/M:** Redistribution of surface LFA-1 on human neutrophils (L) or classical monocytes (M) stimulated for 90 s with CCL5 and pretreated for 30 min with Ac2-26. **N:** GTP-bound Rap1 protein in mouse neutrophils 30 s after stimulation with CCL5 and prestimulation for 30 min with Ac2-26. Representative blots are shown. **O/P:** GTP-bound Rap1 protein in human neutrophils (L) or monocytes (M) 30 s after stimulation with CCL5 and prestimulation for 30 min with Ac2-26. Representative blots and densitometric quantification of one out of four independent experiments is displayed. All data are presented as mean ± SD. Experiments were performed at least 6 times independently for A/B/F/G/H, three times independently for C/D/I/K, and four times independently for L/M. * indicates $p < 0.05$, in F and G * indicates significant difference compared to adhesion in absence of Ac2-26. Data were analyzed using Kruskal-Wallis test with Dunn post test. mu, murine, hu, human.



Online Figure V: In vivo delivery of the Annexin A1 fragment Ac2-26 inhibits arterial leukocyte accumulation. A: *Apoe*$^{-/-}$ mice were fed a high-fat diet for 4 weeks and myeloid cell adhesion was quantified before and at indicated time points after Ac2-26 injection. **B-E:** *Apoe*$^{-/-}$, *Apoe*$^{-/-}$*Fpr2*$^{-/-}$, and *Apoe*$^{-/-}$*Anxa1*$^{-/-}$ mice were fed a high fat diet for 4 weeks. Intravital fluorescence microscopy of the carotid artery for assessment of luminal leukocyte endothelial interactions. Myeloid cell subsets were identified by i.v. injection of antibodies to Ly6G (PE, 0.5 µg) or Ly6C (AF488, 0.5 µg). Adhesion (B and C) and rolling flux (D and E) of Ly6G^{+} cells (B and D) and Ly6C^{+} cells (C and E) were assessed before and 30 minutes after injection of native or boiled Ac2-26 (50 µg, i.v.). **F/G:** All data points represent individual mice. * indicates $p < 0.05$ compared to read-out before Ac2-26 administration. Data were analyzed with either Friedman test with Dunn post-test (A), paired t-test or Wilcoxon matched-pairs signed rank test.